Geneious 7.0

Biomatters Ltd

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Chapter 1

Getting Started

One of the best ways to get an introduction to Geneious, its features and how to use them is to watch our online video demonstration: http://desktop-links.geneious.com/demonstration.

1.1 Downloading & Installing Geneious

Geneious is free to download from http://desktop-links.geneious.com/download. If you are using Geneious for the first time you will be offered a free trial. If you have already purchased a license you can enter it when Geneious starts up.

To download Geneious, click on the internet address above (or type it in to your internet browser) to open the Geneious download page, enter your details, then choose your operating system and click 'Download'. Then choose the version of Geneious you want to download and click "Download" again.

Geneious has some minimum system requirements. It is compatible with the three most common operating systems: Windows, Mac, and Linux. Check that you have one of the following OS versions before you launch Geneious:

Operating System	System requirements
Windows	XP/Vista/7/8
Mac OS	10.6/10.7/10.8
Linux	

Geneious also needs Java 1.6 or higher to run. If you do not have this on your system already, please download a version of Geneious that includes Java. This involves downloading a larger file.

Once Geneious has downloaded, double left-click on the Geneious icon to start installing the program. While this is happening, you will be prompted for a location to install Geneious. Please check that you are satisfied with the location before continuing.

If you are using Mac OS X you will only have to double click on the disk image that is downloaded then drag the Geneious application to your Applications folder. Don't run Geneious from the mounted disk image as there are no write permissions on this. You must drag the icon into your Applications folder and run it from there.

1.1.1 Choosing where to store your data

When Geneious first starts up you will be asked to choose a location where Geneious will store all of your data. The default is normally fine. Although it's possible to store your data on a network or USB drive so you can access it from other computers, this is not recommended because it can have adverse effects on performance. Please do not use a DropBox folder to store your data. This may corrupt your data.

To store your data somewhere different to the default, simply click the 'Select' button in the welcome window and choose an empty folder on your drive where you would like to store your data.

The data location can also be changed later by going to the "General" tab under "Tools" → "Preferences..." in the menu and changing the "Data Storage Location" option. Geneious will offer to copy your existing data across to the new location if appropriate.

1.1.2 Upgrading to new versions

To upgrade existing Geneious installations, simply download and install the new to the same location. This will retain all your data.

1.2 Using Geneious for the first time

Figure 1.1 shows the main Geneious window. This has six important areas or 'panels'.

1.2.1 The Sources Panel

The Sources Panel contains the service Geneious offers for storing and retrieving data. These include your local documents (including sample documents), Shared Databases, UniProt, NCBI, Pfam and Collaboration. All these services will be described in detail later in the manual. For more information see section 2.1.1.

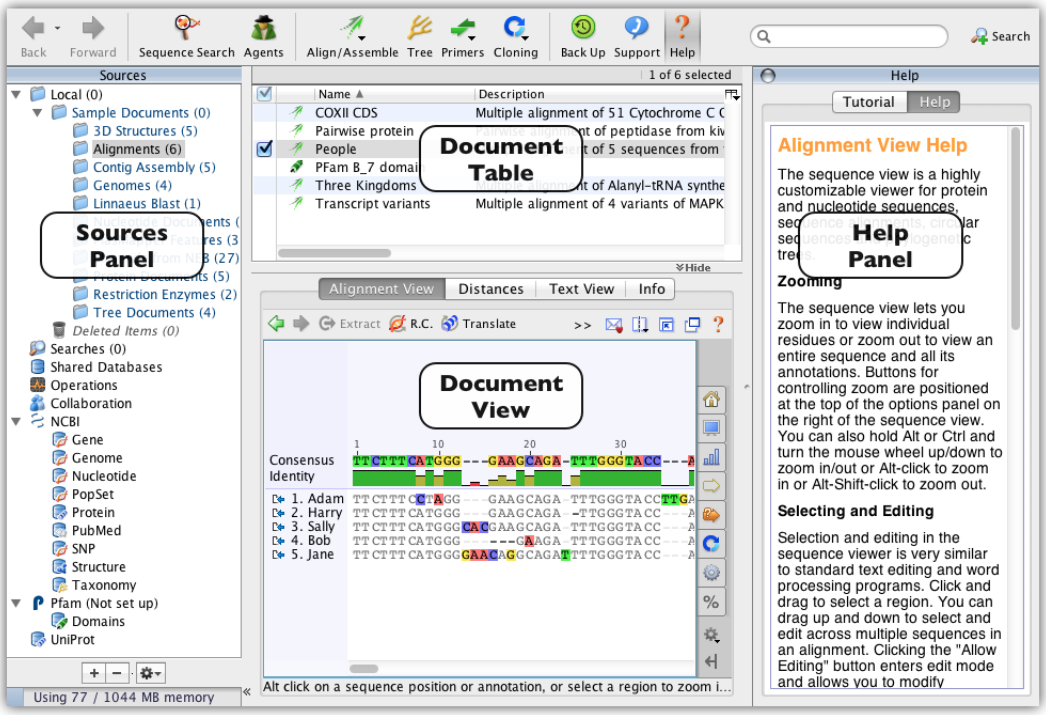


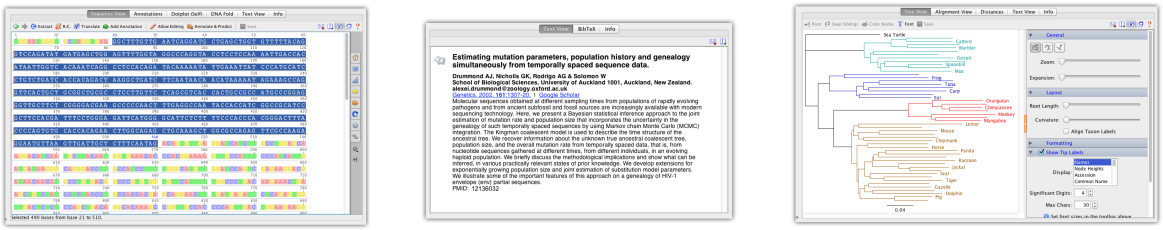
Figure 1.1: The main window in Geneious

1.2.2 The Document Table

The Document Table displays summaries of downloaded data such as DNA sequences, protein sequences, journal articles, sequence alignments, and trees. By clicking on the search icon you can search data for text or by sequence similarity (BLAST). You can enter a search string into the "Filter" box located at the right side of the toolbar; this will hide all documents that do not contain the search string. For more information, see section 2.1.2.

1.2.3 The Document Viewer Panel

The Document Viewer Panel is where sequences, alignments, trees, 3D structures, journal article abstracts and other types of documents can be shown graphically or as plain text. Many document viewers allow you to customize settings such as zoom level, color schemes, layout and annotations (nucleotide and amino acid sequences); three different layouts, branch and leaf labeling (tree documents); and many more. When viewing journal articles, this panel includes direct link to Google Scholar. All these options are displayed on the right-hand side of the panel (Figure 1.2). For more information see section 2.1.3


Estimating mutation parameters, population history and genealogy simultaneously from temporally spaced sequence data.

(a) Nucleotide sequence (b) Journal Article (c) Phylogenetic tree

Figure 1.2: Three document viewers

1.2.4 The Help Panel

The Help Panel has two sections: "Tutorial" and "Help". The tutorial gives you hands-on experience with some of the most popular features of Geneious. The Help section displays a short description of the currently selected service or document viewer. This panel can be closed at any time by clicking the button in its top corner, or by toggling the 'Help' button in the Toolbar.

If you are new to Geneious, working through the tutorial is a great way to familiarize yourself with Geneious.

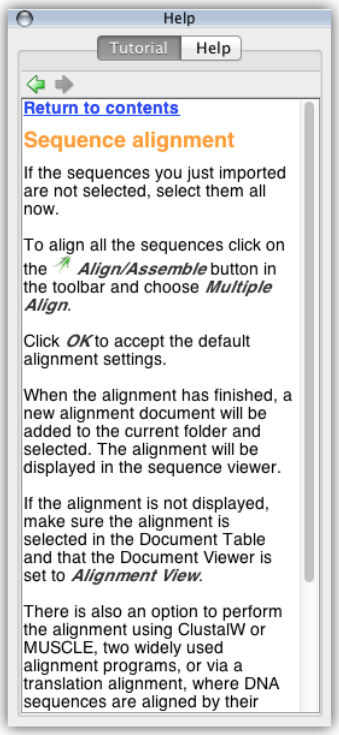

Help
Tutorial
Help
Return to contents
Sequence alignment
If the sequences you just imported are not selected, select them all now.
To align all the sequences click on the Align/Assemble button in the toolbar and choose Multiple Align.
Click OK to accept the default alignment settings.
When the alignment has finished, a new alignment document will be added to the current folder and selected. The alignment will be displayed in the sequence viewer.
If the alignment is not displayed, make sure the alignment is selected in the Document Table and that the Document Viewer is set to Alignment View.
There is also an option to perform the alignment using ClustalW or MUSCLE, two widely used alignment programs, or via a translation alignment, where DNA sequences are aligned by their

Figure 1.3: The Help Panel

1.2.5 The Toolbar

The toolbar gives quick access to commonly used features in Geneious including *Sequence Search* (eg. BLAST), *Agents* that search databases for new content even while you sleep, *Align/Assemble*, *Tree* building, and *Help*. For more information on the toolbar, see section 2.1.5.

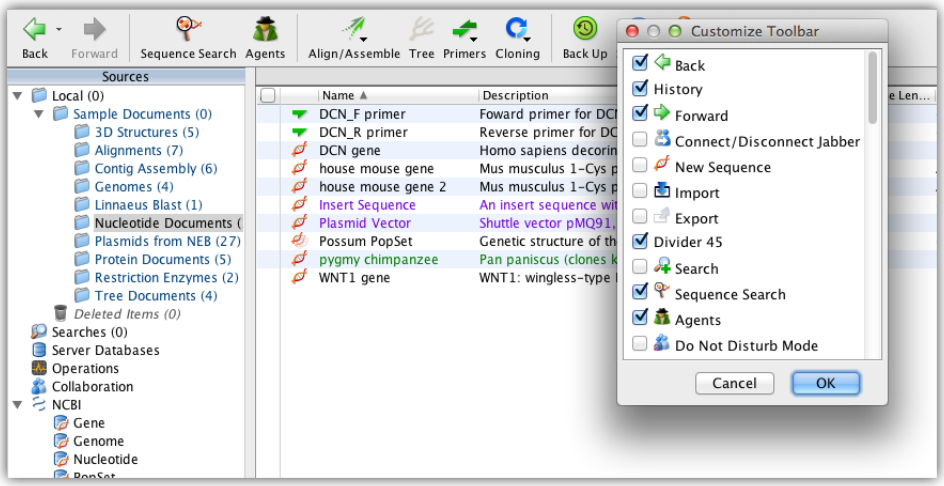


Figure 1.4: The Toolbar

1.2.6 The Menu Bar

The Menu Bar has seven main menus “File”, “Edit”, “View”, “Tools”, “Sequence”, “Annotate & Predict” and “Help”. For details on the menu bar, see section 2.1.7.

1.2.7 Popup Menus

Many actions can be quickly accessed for data items, services and sometimes selections in a viewer via popup menus (also known as *context menus*). To invoke a popup menu for an item, simply right-click (Ctrl+click on Mac OS X). The popup menu will contain the actions which are relevant to the item you clicked.

Chapter 2

Retrieving and Storing data

Geneious is a one-stop-shop for handling and managing your bioinformatic data. This chapter summarizes the different ways you can use Geneious to acquire, update, organize and store your data.

By the end of this chapter, you should be able to:

- Know the purpose of each panel in Geneious
- Import/Export data from various sources
- Organize your data into easily accessible folders
- Automatically update your data
- Know about the advantages of the "Meta-Data" functionality
- Customize Geneious to meet your needs.
- Export and print images from Geneious
- Back up your data

2.1 The main window

This section provides more information on each of the panels in Geneious (Figure 2.1).

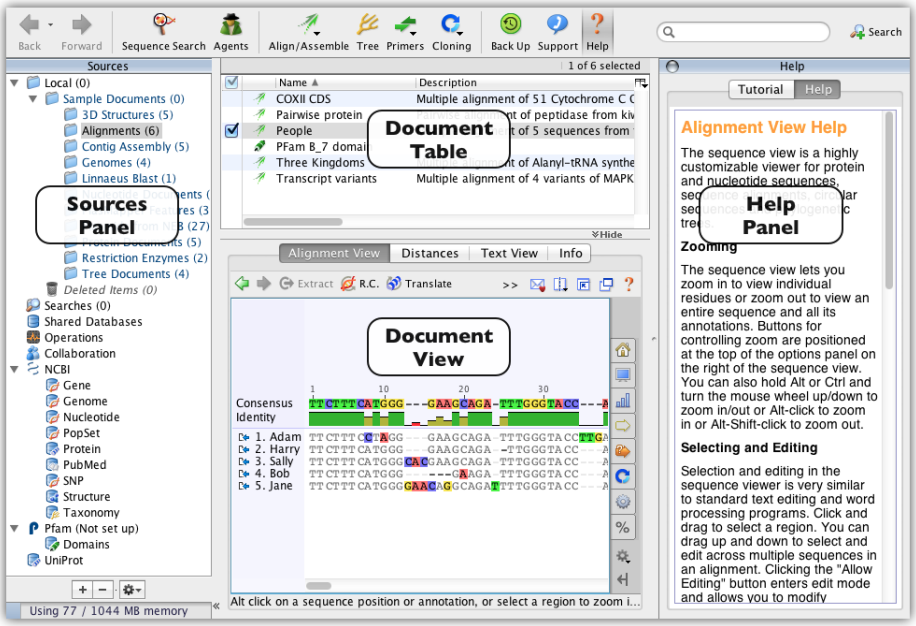


Figure 2.1: Geneious main window

2.1.1 The Sources Panel

The Sources Panel shows a tree that concisely displays sources of data and your stored documents. The plus (+) symbol indicates that a folder contains sub-folders. A minus (-) indicates that the folder has been expanded, showing its sub-folders. Click these symbols to expand or contract folders.

Geneious Sources Panel allows you to access:

- Your Local Documents.
- NCBI databases - Gene, Genome, Nucleotide, PopSet, Protein, Pubmed, SNP, Structure and Taxonomy.
- An EMBL database - Uniprot.
- Your contacts' Geneious databases.

You can view options for any selected service with the right mouse button, or by clicking the Options button at the bottom of the Sources Panel in Mac OS X.

2.1.2 The Documents Table

The Document Table displays your search results or your stored documents. While search results usually contain documents of a single type, a local folder may contain any mixture of documents, whether they are sequences, publications or other types. If you cannot see all of the columns in the document table you may want to close the help panel to make more room.

This information is presented in table form (Figure 2.2).

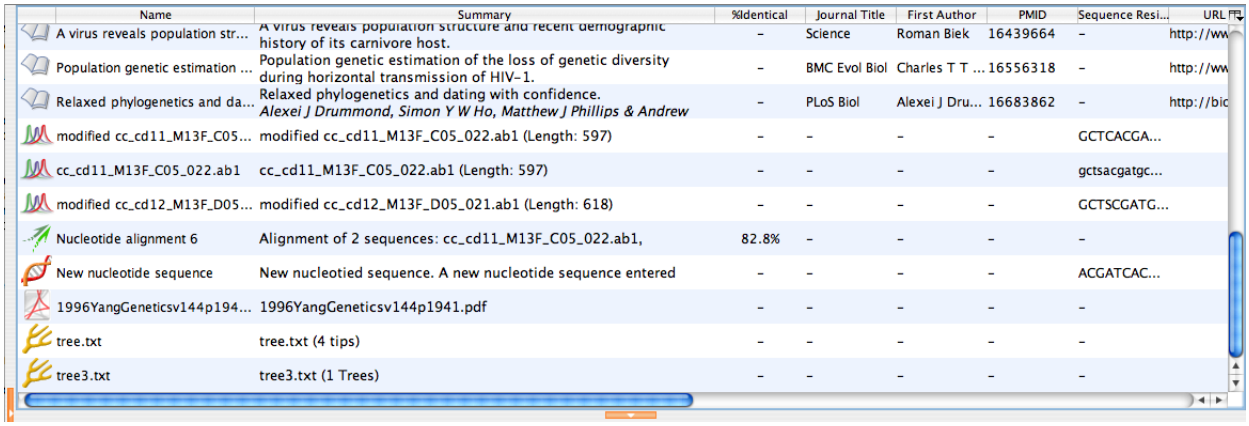


Figure 2.2: The document table, when browsing the local folders

Selecting a document in the Document Table will display its details in the Document View Panel. Selecting multiple documents will show a view of all the selected documents if they are of similar types, e.g. selecting two sequences will show both of them side-by-side in the sequence view.

The easiest way to select multiple documents is by clicking on the checkboxes down the left-hand side of the table. Standard keyboard controls can also be used (Shift and Ctrl/⌘ click).

Double-clicking a document in the Document Table displays the same view in a separate window.

To view the actions available for any particular document or group of documents, right-click (Ctrl+click on Mac OS X) on a selection of them. These options vary depending on the type of document.

The Document Table has some useful features.

Editing. Values can be typed into the columns of the table. This is a useful way of editing the information in a document. To edit a particular value, first click on the document and then click on the column which you want to edit. Enter the appropriate new information and press enter. Certain columns cannot be edited however, eg. the NCBI accession number.

Copying. Column values can be copied. This is a quick method of extracting searchable information such as an accession number. To copy a value, right-click (Ctrl+click on Mac OS X) on it, and choose the "Copy `name`" option, where `name` is the column name.

Sorting. All columns can be alphabetically, numerically or chronologically sorted, depending on the data type. To sort by a given column click on its header. If you have different types of documents in the same folder, click on the "Icon" column to sort then according to their type.

Managing Columns. You can reorder the columns to suit. Click on the column header and drag it to the desired horizontal position.

You can also choose which columns you want to be visible by right-clicking (Ctrl-click on Mac OS X) on any column header or by clicking the small header button in the top right corner of the table. This gives a popup menu with a list of all the available columns. Clicking on a column will show/hide it. Your preference is remembered so if you hide a column it will remain hidden in all areas of the program until you show it again.

As well as items to show/hide any of the available columns, there are a few more options in this popup menu to help you manage columns in Geneious.

- Lock Columns locks the state of the columns in the current table so that Geneious will never modify the way the columns are set up. You can still change the columns your self however.
- Save Current State... allows you to save the the current state if the columns so you can easily apply it to other tables. You can give the state a name and it will then appear in the Load Column State menu.
- Load Column State contains all of the columns states you have saved. Selecting a column state from here will immediately apply that state to the current table and lock the columns to maintain the new state. Use Delete Column State... to remove unwanted columns states from this menu.

Note. New columns can be added to the document table by adding Meta-Data to documents. *(see 2.8 - Meta-Data)*.

2.1.3 The Document Viewer Panel

The Document Viewer Panel shows the contents of any document clicked on in the Document Table. To view large documents, it is sometimes better to double click on them. This opens a view in a new window. In the document viewer panel there are two tabs that are common to most types of documents: "Text view" and "Info". "Text view" shows the document's information in text format. The exception to this rule occurs with PDF documents where the user needs to either click the "View Document" button or double-click to view it.

Some document types such as sequences, trees and structures have an options panel occupying the right of the document viewer. The options in the options panel have an arrow which can be used to expand or hide a group of related options.

See the next section on document viewers for more information about operating the various viewers in Geneious.

Most viewers have their own small toolbar at the top of the document viewer panel. This always has five buttons on the far right:

- "Share" which allows you to share the current visualization on Twitter, Facebook or email.
- "Split View" which opens a second viewer panel of the same document. Selection is synchronized between these two views.
- "Expand Document View" which expands the viewer panel out to fill the entire main window. Clicking again will return the viewer to normal size.
- "Open Document in New Window" will open a new view of the selected document in a new, separate window.
- "Help" opens the Help Panel and displays some short help for the current viewer.

2.1.4 The Help Panel

The Help Panel has a "Help" tab and a "Tutorial" tab.

The Help tab provides you information about the service you are currently using or the viewer you are currently viewing. The help displayed in the help tab changes as you click on different services and choose different viewers.

The Tutorial is aimed at first-time users of Geneious and has been included to provide a feel for how Geneious works. It is highly recommended that you work through the tutorial if you haven't used Geneious before.

2.1.5 The Toolbar

The toolbar contains several icons that provide shortcuts to common functions in Geneious. You can alter the contents of the toolbar to suit your own needs. The icons can be displayed small or large, and with or without their labels. The Help icon is always available.

The "Back" and "Forward" options help you move between previous views in Geneious and are analogous to the back and forward buttons in a web browser. The ▽ option shows a list of

previous views. The other features that can be accessed from the toolbar are described in later sections.

The toolbar can be customized by right-clicking (Ctrl-click on Mac OS X) on it. This gives a popup menu with the following options:

- "Show Labels" Turn the text labels on or off.
- "Large Icons" Switch between large and small icons.
- "Customize" which lists all available toolbar buttons. Selecting/deselecting buttons will show/hide the buttons in the toolbar.

2.1.6 Status bar

Below the Toolbar, there is a grey status bar. This bar displays the status of the currently selected service. For example, when you are running a search, it displays the number of matches, and the time remaining for the search to finish.

2.1.7 The Menu bar

File Menu

This contains some standard "File" menu items including printing and "Exit" on Windows. It also contains options to create, rename, delete, share and move folders and Import/Export options.

Edit Menu

Here you will find common editing functions including "Cut", "Copy", "Paste", "Delete" and "Select All". These are useful when transferring information from within documents to other locations, or exporting them. This menu also contains "Find in Document", "Find Next" and "Find Previous" options. Find can be used to find text or numbers in a selected document. This is useful when looking for annotated regions or a stretch of bases in a sequence. This opens a "Find Dialog". The shortcut to this is Ctrl+F. *Next* finds the next match for the text specified in the "Find" dialog. The shortcut keys are F3 or Ctrl+G. Geneious then allows you to choose another document and continue searching for the same search word. *Prev* finds the previous match. The shortcut keys for this are Ctrl+Shift+G or Shift+F3. There are also the useful "Find Duplicates..." and "Batch Rename..." features in this menu.

View Menu

This contains several options and commands for changing the way you view data in Geneious:

- "Back", "Forwards" and "History" allow you to return to documents you had selected previously.
- "Search" is discussed in section 2.3.
- "Agents" are discussed in section 2.6.
- "Next unread document" selects the next document in the current folder which is unread.
- "Table Columns" contains the same functionality as the popup menu for the document table header. See section 2.1.2 for more details.
- "Open document in new window" Opens a new window with a view of the currently selected document(s).
- "Expand document view" expands the document viewer panel in the main window out to fill the entire main window. Selecting this again to return to normal.
- "Split Viewer Left/Right" creates a second copy of the document viewer with the two views laid out side by side.
- "Split Viewer Top/Bottom" creates a second copy of the document viewer with one on top of the other.
- "Document Windows" Lists the currently open document windows. Selecting one from this menu will bring that document window to the front.

Tools Menu

- "Align/Assemble" - see section 4.4 and section 4.7 respectively
- "Tree" - see section 4.5
- "Primers" - see section 4.6
- "Cloning" - see section 10
- "Sequence Search" - Perform a sequence search (such as NCBI Blast) using the currently selected sequence as the query. See section 2.4.4
- "Add/Remove Databases" - see section 5.1.3
- "Pfam" - see section 7

- "Linnaeus Blast" - Perform a blast search and display the results using the Linnaeus viewer. Evolutionary trees are built for hits within the same species. These are then displayed inside boxes nested according to the NCBI taxonomy.
- "Extract Annotations" - Search the selected sequences or alignments for annotations which match certain criteria then extract all of the matching annotations to separate sequence documents. Includes the option to concatenate all matches in each sequence into one sequence document. Useful for extracting a certain gene from a group of genomes.
- "Strip Alignment Columns" - creates a new alignment document with some columns (for example all identical columns or all columns containing only gaps) stripped
- "Concatenate Sequences or Alignments" - Joins the selected sequences or alignments end-on-end, creating a single sequence or alignment document from several. After selecting this operation you are given the option to choose the order in which the sequences or alignments are joined. You can also choose whether the resulting document is linear or circular, and, if one or more of the component sequences was an extraction from over the origin of a circular sequence, you can choose to use the numbering from that sequence, thus producing a circular sequence with its origin in the same place as the original circular sequence. Overhangs will be taken into account when concatenating.
- "Generate Consensus Sequence" - Generates a consensus sequence for the selected sequence alignment and saves it to a separate sequence document. After selecting this operation you are given options for choosing what type of consensus sequence you wish to generate - see section 3.2.6 for more details on the options.
- "Plugins" - Jump directly to the plugins preferences.
- "Preferences" - see section 2.9

2.1.8 Sequence Menu

This contains several operations that can be performed on Protein and Nucleotide sequences as well as Sequence Alignments in some cases.

- *New Sequence* create a new nucleotide or protein sequence from residues that you can paste or type in.
- *Extract Region*, *Reverse Complement*, *Translate*. Sometimes a selection in the sequence viewer is required before performing these.
- *Back Translate* creates an ambiguous nucleotide version of the selected protein document.
- *Circular Sequences* sets whether the currently selected sequences are circular. This effects the way the sequence view displays them as well as how certain operations deal with the sequences (eg. digestion).

- *Free End Gaps Alignment* sets whether the currently selected alignment has free end gaps. This effects calculation of the consensus sequences and statistics.
- *Change Residue Numbering...* changes the "original residue numbering" of the selected sequence. On a linear sequence, this is used to indicate that a sequence is a subsequence of some larger sequence. On a circular sequence, this is used to shift the origin of the sequence.
- *Convert between DNA and RNA* changes all T's in a sequence to U's or vice versa, depending on the type of the selected sequence. Once this is performed, click "Save" in the Sequence View to make the change permanent.
- *Set Paired Reads* sets up paired reads for assembly. See section 4.7.4
- *Set Read Direction* marks sequences as forward or reverse reads so the correct reads are reverse complemented by assembly.
- *Separate Reads by Barcode* separates multiplex or barcode data (e.g. 454 MID data).
- *Group Sequences into a List* creates a sequence list containing copies of all of the selected sequences. Lists can make it easier to manager large numbers of sequences by keeping related ones grouped in a single document.
- *Extract Sequence from List* copies each sequence out of a sequence list into a separate sequence document.
- *Generate Mutated Sequences* mutates a sequence using the EMBOSS tool msbar
- *Generate Shuffled Sequences* randomly shuffles a sequence using the EMBOSS tool shuffleseq

2.1.9 Annotate & Predict Menu

This menu contains many tools for finding, predicting and annotating regions of interest in sequences and alignments.

- *Trim Ends* See section 4.7.3.
- *Annotate from Database* Annotates sequences with similar annotations from your database.
- *Find ORFs* Finds all open reading frames in a sequence and annotates them
- *Search for Motifs* searches for motifs in PROSITE format. Uses "fuzznuc" and "fuzzpro" from EMBOSS.
- *Find Variations/SNPs* finds variable positions in assemblies and alignments

- *Find Low/High Coverage* finds regions with low or high read coverage in assemblies
- *Download Annotation Tracks* annotates chromosomes with tracks from the Broad Institute
- *Search for Transcription Factors* searches for transcription factors from the TRANSFAC database in a nucleotide sequence. Uses "tfscan" from EMBOSS
- *Predict Antigenic Regions* predicts potentially antigenic regions of a protein sequence, using the method of Kolaskar and Tongaonkar. Uses "antigenic" from EMBOSS
- *Predict Secondary Structure* uses the original Garnier Osguthorpe Robson algorithm (GOR I) for predicting protein secondary structure. Uses "garnier" from EMBOSS
- *Predict Signal Cleavage Sites* predicts the site of cleavage between a signal sequence and the mature exported protein. Uses "sigcleave" from EMBOSS

Help Menu

This consists of the standard Help options offered by Geneious.

- *Help* shows and hides the Help panel
- *Tutorial* shows and hides the Tutorial panel
- *Online Resources*gives access to a variety of resources on our website
- *Check for Updates* checks for new versions of Geneious
- *Contact Support* allows you to contact our Support team through Geneious
- *Activate License* lets you activate a license or connect to a license server
- *Install FLEXnet* installs the FLEXnet licensing service which is necessary to use FLEXnet licenses
- *Borrow Floating License* lets you borrow a license from a FLEXnet server, if the maintainer of the server has provided you with a Borrow File
- *Release Licenses* releases any floating license you are currently holding and returns any local FLEXnet licenses to our server so they can be activated on a difference machine
- *Buy Online* sends you to our online store
- *About Geneious* gives details about the version of Geneious you are running, and licensing information

2.2 Importing and exporting data

Geneious is able to import raw data from different applications and export the results in a range of formats. If you are new to bioinformatics, please take the time to familiarize yourself with this chapter as there are a number of formats to be aware of.

2.2.1 Importing data from the hard drive to your Local folders

To import files from local disks or network drives, click "File" → "Import" → "From file". This will open up a file dialog. Select one or more files and click "Import". If Geneious' automatic file format detection fails, select the file type you wish to import (Figure 2.3). The different file types are described in detail in the next section.

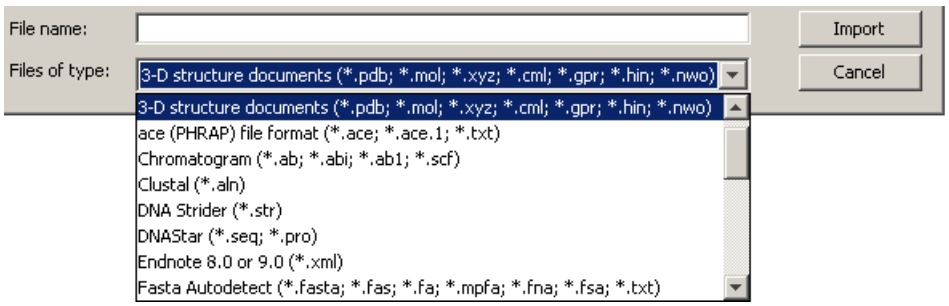

File name:
Import
Files of type:
3-D structure documents (*.pdb; *.mol; *.xyz; *.cml; *.gpr; *.hin; *.nwo)
Cancel
3-D structure documents (*.pdb; *.mol; *.xyz; *.cml; *.gpr; *.hin; *.nwo)
ace (PHRAP) file format (*.ace; *.ace.1; *.txt)
Chromatogram (*.ab; *.abi; *.ab1; *.scf)
Clustal (*.aln)
DNA Strider (*.str)
DNAStar (*.seq; *.pro)
Endnote 8.0 or 9.0 (*.xml)
Fasta Autodetect (*.fasta; *.fas; *.fa; *.mpfa; *.fna; *.fsa; *.txt)

Figure 2.3: File import options

2.2.2 Data input formats

Geneious version 7.0 can import the following file formats:

Format	Extensions	Data types	Common sources
BED	*.bed	Annotations	UCSC
Common Assembly Format	*.caf	Contigs	Sequencher
Clustal	*.aln	Alignments	ClustalX
CSFASTA	*.csfasta	Color space FASTA	ABI SOLiD
DNAStar	*.seq, *.pro	Nucleotide & protein sequences	DNAStar
DNA Strider	*.str	Sequences	DNA Strider (Mac program), ApE
Embl/UniProt	*.embl, *.swp	Sequences	Embl, UniProt
Endnote (8.0) XML	*.xml	Journal article references	Endnote, Journal article websites
FASTA	*.fasta, *.fas, etc.	Sequences, alignments	PAUP*, ClustalX, BLAST, FASTA
FASTQ	*.fastq, *.fasq	Sequences with quality	Solexa/Illumina
GCG	*.seq	Sequences	GCG
GenBank	*.gb, *.xml	Nucleotide & protein sequences	GenBank
Geneious	*.xml, *.geneious	Preferences, databases	Geneious
Geneious Education	*.tutorial.zip	Tutorial, assignment etc.	Geneious
GFF	*.gff	Annotations	Sanger Artemis
MEGA	*.meg	Alignments	MEGA
Molecular structure	*.pdb, *.mol, *.xyz, *.cml, *.gpr, *.hin, *.nwo	3D molecular structures	3D structure databases and programs
Newick	*.tre, *.tree, etc.	Phylogenetic trees	PHYLIP, Tree-Puzzle, PAUP*, ClustalX
Nexus	*.nxs, *.nex	Trees, Alignments	PAUP*, Mesquite, MrBayes & MacClade
PDB	*.pdb	3D Protein structures	SP3, SP2, SPARKS, Protein Data Bank
PDF	*.pdf	Documents, presentations	Adobe Writer, LaTeX, Miktex
Phrap ACE	*.ace	Contig assemblies	Phrap/Consed
PileUp	*.msf	Alignments	pileup (gcg)
PIR/NBRF	*.pir	Sequences, alignments	NBRF PIR
Qual	*.qual	Quality file	Associated with a FASTA file
Raw sequence text	*.seq	Sequences	Any file that contains only a sequence
Rich Sequence Format	*.rsf	Sequences, alignments	GCGs NetFetch
Comma/Tab Separated Values	*.csv, *.tsv	Spreadsheet files	Microsoft Excel
SAM/BAM	*.sam, *.bam	Contigs	SAMtools
Sequence Chromatograms	*.ab1, *.scf	Raw sequencing trace & sequence	Sequencing machines
VCF	*.VCF	Annotations	1000 Genomes Project
Vector NTI sequence	*.gb, *.gp	Nucleotide & protein sequences	Vector NTI
Vector NTI/AlignX alignment	*.apr	Alignments	Vector NTI, AlignX
Vector NTI Archive	*.ma4, *.pa4, *.oa4, *.ea4, *.ca6	Nucleotide & protein sequences, enzyme sets and publications	Vector NTI
Vector NTI/ContigExpress	*.cep	Nucleotide sequence assemblies	Vector NTI
Vector NTI database	VNTI Database	Nucleotide & protein sequences, enzyme sets and publications	Vector NTI

BED format

The BED format contains sequence annotation information. You can use a BED file to annotate existing sequences in your local database, import entirely new sequences, or import the annotations onto blank sequences.

CLUSTAL format

The Clustal format is used by ClustalW [24] and ClustalX [23], two well known multiple sequence alignment programs.

Clustal format files are used to store multiple sequence alignments and contain the word clustal at the beginning. An example Clustal file:

```
CLUSTAL W (1.74) multiple sequence alignment
```

```
seq1 ----------------------KSKERYKDENGGNYFQLREDWWDANRETVWKAITCNA
seq2 ---------------YEGLTTANGXKEYYQDKNGGNFFKLREDWWTANRETVWKAITCGA
seq3 ----KRIYKKIFKEIHSGLSTKNGVKDRYQN-DGDNYFQLREDWWTANRSTVWKALTCSD
seq4 ------------------------SQRHYKD-DGGNYFQLREDWWTANRHTVWEAITCSA
seq5 --------------------NVAALKTRYEK-DGQNFYQLREDWWTANRATIWEAITCSA
seq6 ------FSKNIX--QIEELQDEWLLEARYKD--TDNYYELREHWWTENRHTVWEALTCEA
seq7 -------------------------------------------------KELWEALTCSR

seq1 --GGGKYFRNTCDG--GQNPTETQNNCRCIG----------ATVPTYFDYVPQYLRWSDE
seq2 P-GDASYFHATCDSGDGRGGAQAPHKCRCDG---------ANVVPTYFDYVPQFLRWPEE
seq3 KLSNASYFRATC--SDGQSGAQANNYCRCNGDKPDDDKP-NTDPPTYFDYVPQYLRWSEE
seq4 DKGNA-YFRRTCNSADGKSQSQARNQCRC---KDENGKN-ADQVPTYFDYVPQYLRWSEE
seq5 DKGNA-YFRATCNSADGKSQSQARNQCRC---KDENGXN-ADQVPTYFDYVPQYLRWSEE
seq6 P-GNAQYFRNACS----EGKTATKGKCRCISGDP----------PTYFDYVPQYLRWSEE
seq7 P-KGANYFVYKLD-----RPKFSSDRCGHNYNGDP---------LTNLDYVPQYLRWSDE
```

CSFASTA format

ABI .csfasta files represent the color calls generated by the SOLiD sequencing system.

DNAStar files

DNAStar .seq and .pro files are used in Lasergene, a sequence analysis tool produced by DNAStar.

DNA Strider

Sequence files generated by the Mac program DNA Strider, containing one Nucleotide or Protein sequence.

EMBL/UniProt

Nucleotide sequences from the EMBL Nucleotide Sequence Database, and protein sequences from UniProt (the Universal Protein Resource)

EndNote 8.0 XML format

EndNote is a popular reference and bibliography manager. EndNote lets you search for journal articles online, import citations, perform searches on your own notes, and insert references into documents. It also generates a bibliography in different styles. Geneious can interoperate with EndNote using Endnote's XML (Extensible Markup Language) file format to export and import its files.

FASTA format

The FASTA file format is commonly used by many programs and tools, including BLAST [1], T-Coffee [17] and ClustalX [23]. Each sequence in a FASTA file has a header line beginning with a ">" followed by a number of lines containing the raw protein or DNA sequence data. The sequence data may span multiple lines and these sequence may contain gap characters. An empty line may or may not separate consecutive sequences. Here is an example of three sequences in FASTA format (DNA, Protein, Aligned DNA):

```
>Orangutan
ATGGCTTGTGGTCTGGTCGCCAGCAACCTGAATCTCAAACCTGGAGAGTGCCTTCGAGTG

>gi|532319|pir|TVFV2E|TVFV2E envelope protein
ELRLRYCAPAGFALLKCNDADYDGFKTNCSNVSVVHCTNLMNTTVTTGLLLNGSYSENRT
QIWQK

>Chicken
CTACCCCCCTAAAACACTTTGAAGCCTGATCCTCACTA------------------CTGT
CATCTTAA
```

FASTQ format

FASTQ format stores sequences and Phred qualities in a single file.

GenBank files

Records retrieved from the NCBI webiste (http://www.ncbi.nlm.nih.gov) can be saved in a number of formats. Records saved in GenBank or INSDSeq XML formats can be imported into Geneious.

Geneious format

The Geneious format can be used to store all your local documents, meta-data types and program preferences. A file in Geneious format will usually have a `.geneious` extension or a `.xml` extension. This format is useful for sharing documents with other Geneious users and backing up your Geneious data.

Geneious Education format

This is an archive containing a whole bundle of files which together comprise a Geneious education document. This format can be used to create assignments for your students, bioinformatics tutorials, and much more. See chapter 8 for information on how to create such files.

GFF format

The GFF format contains sequence annotation information (and optional sequences). You can use a GFF file to annotate existing sequences in your local database, import entirely new sequences, or import the annotations onto blank sequences.

MEGA format

The MEGA format is used by MEGA (Molecular Evolutionary Genetics Analysis).

Molecular structure

Geneious imports a range of molecular structure formats. These formats support showing the locations of the atoms in a molecule in 3D:

- **PDB format** files from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Database
- ***.mol format** files produced by MDL Information Systems Inc
- ***.xyz format** files produced by XMol
- ***.cml format** files in Chemical Markup Language
- ***.gpr format** ghemical files
- ***.hin format** files produced by HyperChem
- ***.nwo format** files produced by NWChem

Newick format

The Newick format is commonly used to represent phylogenetic trees (such as those inferred from multiple sequence alignments). Newick trees use pairs of parentheses to group related taxa, separated by a comma (,). Some trees include numbers (branch lengths) that indicate the distance on the evolutionary tree from that taxa to its most recent ancestor. If these branch lengths are present they are prefixed with a colon (:). The Newick format is produced by programs such as PHYLIP, PAUP*, ClustalW [24], ClustalX [23], Tree-Puzzle [8] and PROTML. Geneious is also able to read trees in Newick format and display them in the visualization window. It also gives you a number of display options including tree types, branch lengths, and labels.

Nexus format

The Nexus format [13] was designed to standardize the exchange of phylogenetic data, including sequences, trees, distance matrices and so on. The format is composed of a number of blocks such as TAXA, TREES and CHARACTERS. Each block contains pre-defined fields. Geneious imports and exports files in Nexus format, and can process the information stored in them for analysis.

If you want to export a tree in a format that preserves **bootstrap** values for example, Nexus is the choice. Make sure you export with metacomments enabled though otherwise the bootstraps will be lost.

PDB format

Protein Databank files contain a list of XYZ co-ordinates that describe the position of atoms in a protein. These are then used to generate a 3D model which is usually viewed with Rasmol or SPDB viewer. Geneious can read PDB format files and display an interactive 3D view of the protein structure, including support for displaying the protein's secondary structure when the appropriate information is available.

PDF format

PDF stands for Portable Document Format and is developed and distributed by Adobe Systems (`http://www.adobe.com/`). It contains the entire description of a document including text, fonts, graphics, colors, links and images. The advantage of PDF files is that they look the same regardless of the software used to create them. Some word processors are able to export a document into PDF format. Alternatively, Adobe Writer can be used. Currently, you can use Geneious to read, store and open PDF files and future versions will have more options for storing and manipulating PDF.

Phrap Ace files

Ace is the format used by the Phrap/Consed package, created by the University of Washington Genome Center. This package is used mainly to assemble sequences.

PileUp format

The PileUp format is used by the pileup program, a part of the Genetics Computer Group (GCG) Wisconsin Package.

PIR/NBRF format

Format used by the Protein Information Resource, a database established by the National Biomedical Research Foundation

Qual file

Quality file which must be in the same folder as the sequence file (FASTA format) for the quality scores to be used.

Raw sequence format

A file containing only a sequence

Rich Sequence format

RSF (Rich Sequence Format) files contain one or more sequences that may or may not be related. In addition to the sequence data, each sequence can be annotated with descriptive sequence information.

Comma/Tab Separated Values

Sequences such as primer lists are often stored in spreadsheets. Geneious has an importer that can be given the field values for a spreadsheet file exported in CSV or TSV format, and it will import them and convert them to documents as well as preserving the additional field contents. It can handle nucleotide and amino acid sequences, as well as primers and probes. For more information on importing primers from a spreadsheet, see the PCR Primers section.

SAM and BAM format

SAM and BAM format are produced and used by SAMtools. SAM/BAM files contain the results of an assembly in the form of reads and their mappings to reference sequences.

Sequence Chromatograms

Sequence chromatogram documents contain the results of a sequencing run (the trace) and a guess at the sequence data (base calling).

Informally, the trace is a graph showing the concentration of each nucleotide against sequence positions. Base calling software detects peaks in the four traces and assigns the most probable base at more or less even intervals.

VCF format

The VCF format contains sequence annotation information. You can use a VCF file to annotate existing sequences in your local database, import entirely new sequences, or import the annotations onto blank sequences.

Vector NTI® formats

Geneious supports the import of several Vector NTI formats:

- ***.gb and *.gp formats** These formats are used in Vector NTI for saving single nucleotide and protein sequence documents. They are very similar to the GenBank formats with the same extensions, although they contain some extra information.
- ***.apr format** This format is used for storing alignments and trees made with AlignX, Vector NTI's alignment module.
- ***.ma4, *.pa4, *.oa4, *.ea4 and *.ca6 formats** These are the archive formats which Vector NTI uses to export whole databases.
- ***.cep format** This format is produced by the ContigExpress module and Geneious will import sequences (including the positions of the base calls), traces, qualities, trimmed regions, annotations and editing history for individual reads and contigs.

2.2.3 Where does my imported data go?

The above formats can be all imported into Geneious from local files. Geneious also enables you to download certain types of documents directly from public databases such as NCBI and EMBL. The method used to retrieve a particular piece of data will determine where in Geneious it is stored.

Data imported from local files. This is imported directly into the currently selected local folder within Geneious. If no folder is selected, Geneious will open a dialog which lets you specify a folder.

Data from an NCBI/EMBL/Contacts search. Data downloaded from public databases within Geneious will appear in the Document Table when that database is selected and can be dragged from there into a local folder of your choice.

Important: if you don't drag the documents from a database search into your local folders the results will be lost when Geneious is closed.

2.2.4 Data output formats

Each data type has several export options. Any set of documents may be exported in Geneious native format.

Data type	Export format options
DNA sequence	FASTA, Genbank XML, Genbank flat, Geneious
Amino acid sequence	FASTA, Genbank XML, Genbank flat, Geneious
Chromatogram sequence	ABI, Geneious
Sequence with quality	FastQ, Qual, Geneious
Annotation	GFF, BED, Geneious
Multiple sequence alignment	Phylip, FASTA, NEXUS [13], MEGA3 [12], Geneious
Assembly	Phrap ACE, Geneious, SAM/BAM
Phylogenetic tree	Phylip, FASTA, NEXUS [13], Newick, MEGA3 [12], Geneious
PDF document	PDF, Geneious
Publication	EndNote 8.0, Geneious
Graphs, CSV, WIG	
Document Properties	CSV, TSV, Geneious

Additionally, documents imported in any chromatogram or molecular structure format can be re-exported in that format as long as no changes have been made to the document.

2.2.5 Export to comma separated values (CSV) file

The value displayed in the document table can be exported to csv file which can be loaded by most spread sheet programs. When choosing to export in csv format Geneious will also present a list of the available columns in the table (including hidden ones) so you can choose which to export. Data can be exported to TSV (tab separated values) format too.

There is also a CSV importer. It is often useful to export your data to a spreadsheet to do bulk modifications to fields and then reimport.

2.2.6 Batch Export

Batch export takes the selected documents and exports each to its own file. E.g. select several chromatograms to export them all to ab1 format files. The options for batch export let you specify the format and folder to export to as well as the extension to use. Each file will be named according to the Name column in Geneious.

2.3 Searching

Searching is designed to be as user-friendly as possible and the process is the same if you are searching your local documents or a public database such as NCBI. To search the selected database or folder click the "Search" button from the toolbar. For non-local folders search will be on by default and cannot be closed. This applies to NCBI and EMBL databases. For local folders search is off by default.

When search is first activated the document table will be emptied to indicate no results have been found. To return to browsing click the "Search" button again or press the Escape key while the cursor is in the search text field.

To initiate a search enter the desired search term(s) in the text field and press enter or click the adjacent "Search" button. Once a search starts the results will appear in the document table as they are found. The "Search" button changes to a "Cancel" button while a search is in progress and this may be clicked at any time to terminate the search. Feedback on a search progress is presented in the status bar directly below the toolbar (see Figure 2.4).

2.3.1 Advanced Search options

To access advanced search click the "More Options" button inside the basic search panel. To return to basic search click the "Fewer Options" button. Switching between advanced and basic will not clear the search results table.

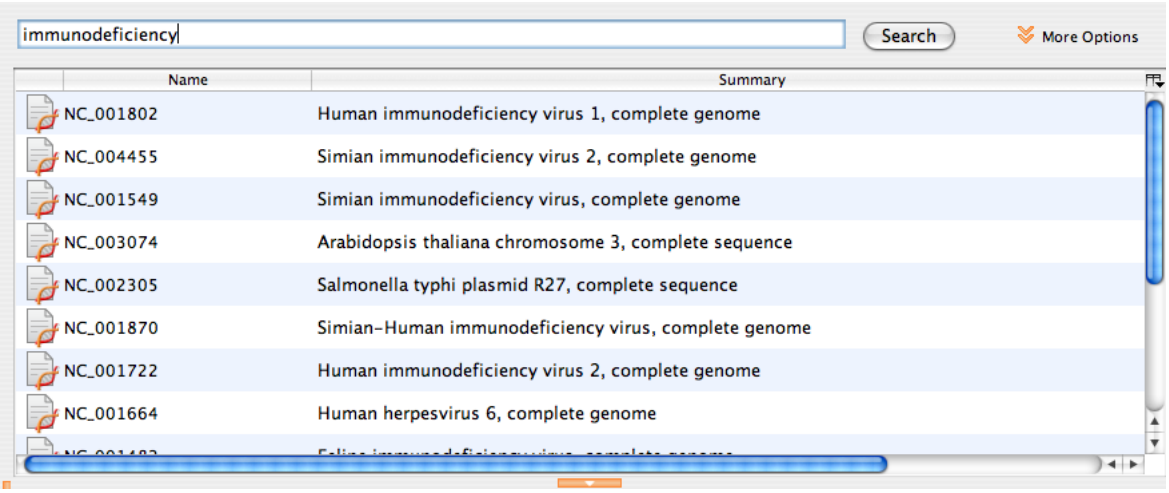


Figure 2.4: The Search tab of the Document Table

This feature provides more search options to select from. Geneious allows you to search with a range of criteria; however, these depend on the database being searched. All the fields in the NCBI public databases can be searched in any combination. Each database has a specific list of fields and it is important to familiarize yourself with these fields to make full use of the Advanced Search. The fields available for a search can be found in the left-most drop-down box after enabling the advanced search options.

When searching in your local documents, '?' can be used to represent any single character and '*' can be used to represent a series of 0 or more unknown characters. For example, searching for CO*I matches COI and COXI.

Note. When searching the Genome, Gene or PopSet databases, the documents returned are only summaries. To download the whole genome, select the summary(s) of the genome(s) you would like to download and the click the "Download" button inside the document view or just above it. There are also "Download" items in the File menu and in the popup menu when document summary is right-clicked (Ctrl+click on Mac OS X). The size of these files is not displayed in the Documents Table. Be aware that whole genomes can be very large and can take a long time to download. You can cancel the download of document summaries by selecting "Cancel Downloads" from any of the locations mentioned above.

Advanced Search also provides you with a number of options for restricting the search on a field depending on the field you are searching against. For example, if you are using numbers to search for "Sequence length" or "No. of nodes" you can further restrict your search with the second drop-down box:

- "is greater than" (>)

- "is less than" ($<$)
- "is greater than or equal to" ($\geq$)
- "is less than or equal to" ($\leq$)

Likewise if you are searching on the "Creation Date" search field you have the following options

- "is before or on"
- "is after or on"
- "is between"

When searching your local folders you have the option of searching by "Document type". The second drop-down list provides the options "is" and "is not". The third drop-down lists the various types of documents that can be stored in Geneious such as "3D-Structure", "Nucleotide sequence", and "PDF" (see Figure 2.5).

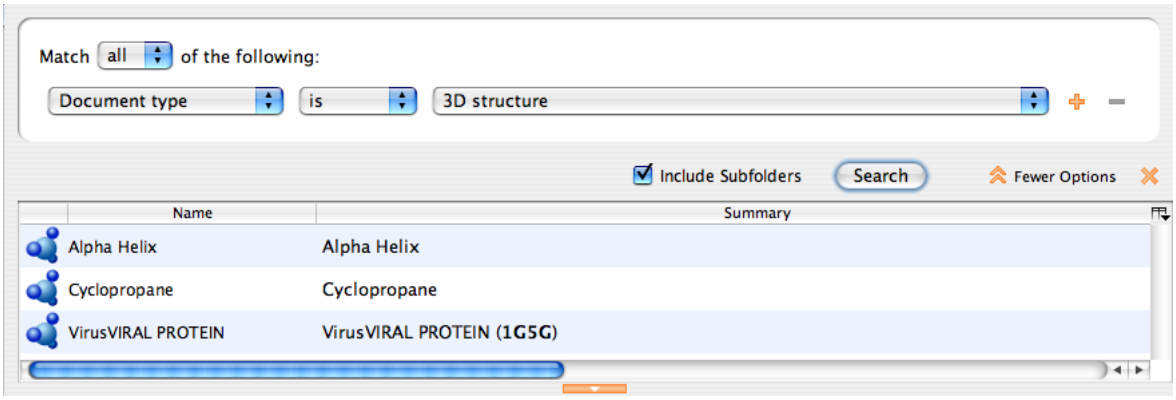


Figure 2.5: Document type search options

And/Or searches

The advanced options lets you search using multiple criteria. By clicking the "+" button on right of the search term you can add another search criteria. You can remove search criteria by clicking on the appropriate "-" button. The "Match all/any of the following" option at the top of the search terms determines how these criteria are combined:

Match "Any" requires a match of one or more of your search criteria. This is a broad search and results in more matches.

Match "All" requires a match all of your search criteria. This is a narrow search and results in fewer matches.

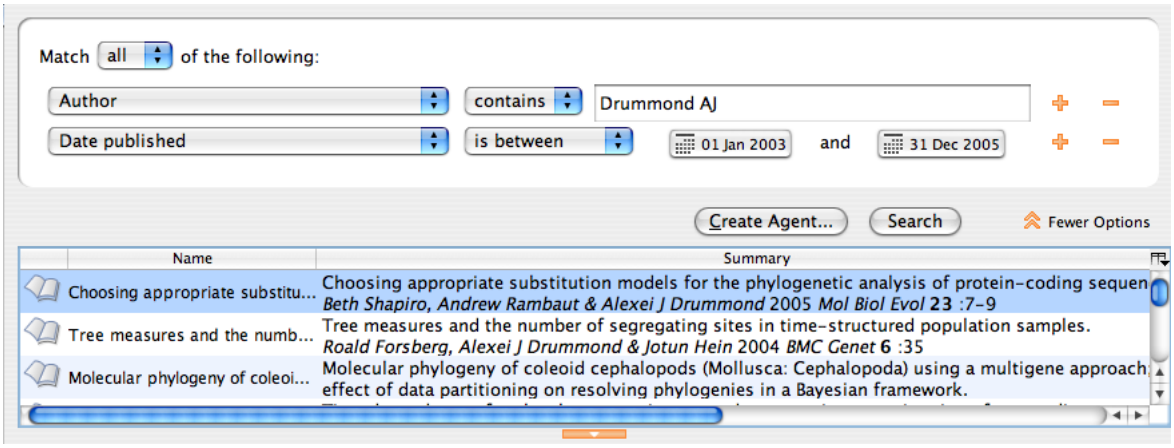

Match all of the following:
Author
contains
Drummond AJ
Date published
is between
01 Jan 2003
and
31 Dec 2005
Create Agent...
Search
Fewer Options
Name
Summary
Choosing appropriate substitu...
Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequen
Beth Shapiro, Andrew Rambaut & Alexei J Drummond 2005 Mol Biol Evol 23 :7-9
Tree measures and the numb...
Tree measures and the number of segregating sites in time-structured population samples.
Roald Forsberg, Alexei J Drummond & Jotun Hein 2004 BMC Genet 6 :35
Molecular phylogeny of coleoi...
Molecular phylogeny of coleoid cephalopods (Mollusca: Cephalopoda) using a multigene approach
effect of data partitioning on resolving phylogenies in a Bayesian framework.

Figure 2.6: Advanced Search

2.3.2 Autocompletion of search words

Geneious remembers previously searched keywords and offers an auto-complete option. This works in a similar way to Google or predictive text on your mobile phone. If you click within the search field, a drop-down box will appear showing previously used options.

2.4 Public databases

Geneious allows you to search several public databases in the same way that you can search your local documents. The search process is described in section 2.3.

Geneious is able to communicate with a number of public databases hosted by the National Centre for Biotechnology Information (NCBI) as well as the UniProt and Pfam databases. You can access these databases through the web at `http://www.ncbi.nlm.nih.gov`, `http://www.uniprot.org/` and `http://www.sanger.ac.uk/Software/Pfam/` respectively. These are all well known and widely used storehouses of molecular biology data.

When viewing data from a public database such as NCBI the data can not be modified. This is demonstrated by the small padlock icon which appears in the status bar. When this icon is present items cannot be added or removed from the table and they cannot be modified in any way. To modify an item you must first move it to your local folders.

2.4.1 Pfam

See chapter 7.

2.4.2 UniProt

This database is a comprehensive catalogue of protein data. It includes protein sequences and functions from Swiss-Prot, TrEMBL, and PIR.

2.4.3 NCBI (Entrez) databases

NCBI was established in 1988 as a public resource for information on molecular biology. Geneious allows you to directly download information from nine important NCBI databases and perform NCBI BLAST searches (Table 2.1).

Table 2.1: NCBI databases accessible via Geneious

Database	Coverage
Genome	Whole genome sequences
Nucleotide	DNA sequences
PopSet	sets of DNA sequences from population studies
Protein	Protein sequences
Structure	3D structural data
PubMed	Biomedical literature citations and abstracts
Taxonomy	Names and taxonomy of organisms
SNP	Single Nucleotide Polymorphisms
Gene	Genes

The Entrez Genome database. The Entrez genome database has been retired. For backwards compatibility Geneious simulates searching of the old genome database by searching the Entrez Nucleotide database and filtering the results to include only genome results.

The Entrez Nucleotide database. This database in GenBank contains 3 separate components that are also searchable databases: "EST", "GSS" and "CoreNucleotide". The core nucleotide database brings together information from three other databases: GenBank, EMBI, and DDBJ. These are part of the International collaboration of Sequence Databases. This database also contains RefSeq records, which are NCBI-curated, non-redundant sets of sequences.

The Entrez Popset database. This database contains sets of aligned sequences that are the result of population, phylogenetic, or mutation studies. These alignments usually describe evolution

and population variation. The PopSet database contains both nucleotide and protein sequence data, and can be used to analyze the evolutionary relatedness of a population.

The Entrez Protein database. This database contains sequence data from the translated coding regions from DNA sequences in GenBank, EMBL, and DDBJ as well as protein sequences submitted to the Protein Information Resource (PIR), SWISS-PROT, Protein Research Foundation (PRF), and Protein Data Bank (PDB) (sequences from solved structures).

The Entrez Structure database. This is NCBI's structure database and is also called MMDB (Molecular Modeling Database). It contains three-dimensional, biomolecular, experimentally or programmatically determined structures obtained from the Protein Data Bank.

The PubMed database. This is a service of the U.S. National Library of Medicine that includes over 16 million citations from MEDLINE and other life science journals. This archive of biomedical articles dates back to the 1950s. PubMed includes links to full text articles and other related resources, with the exception of those journals that need licenses to access their most recent issues.

Entrez Taxonomy. This database contains the names of all organisms that are represented in the NCBI genetic database. Each organism must be represented by at least one nucleotide or protein sequence.

Entrez Gene. Entrez Gene is NCBI's database for gene-specific information. It does not include all known or predicted genes; instead Entrez Gene focuses on the genomes that have been completely sequenced, that have an active research community to contribute gene-specific information, or that are scheduled for intense sequence analysis.

Entrez SNP. In collaboration with the National Human Genome Research Institute, The National Center for Biotechnology Information has established the dbSNP database to serve as a central repository for both single base nucleotide subsitutions and short deletion and insertion polymorphisms.

The scope and depth of these databases make them critical information sources for molecular biologists and bioinformaticians alike. However, a library is only as good as its librarian. Geneious is your librarian, allowing you to search for, filter and store, only the data that you care about.

2.4.4 Accessing NCBI BLAST through Geneious

BLAST [1] stands for Basic Local Alignment Search Tool. It allows you to query the NCBI sequence databases with a sequence in order to find entries in the public database that contain similar sequences. When "BLAST-ing", you are able to specify either nucleotide or protein sequences and nucleotide sequences can be either DNA or RNA sequences. The result of a BLAST query is a table of "hits". Each hit refers to a GenBank accession number and the gene or protein name of the sequence. Each hit also has a "Bit-score" which provides information

about how similar the hit is to the query sequence. The bigger the bit score, the better the match. Finally there is also an "E-value" or "Expect value", which represents the number of hits with at least this score that you would expect purely by chance, given the size of the database and query sequence. The lower the E-value, the more likely that the hit is real.

Geneious can perform seven different kinds of BLAST search:

- **blastn**: Compares a nucleotide query sequence against a nucleotide sequence database.
- **Megablast**: A variation on blastn that is faster but only finds matches with high similarity.
- **Discontiguous Megablast**: A variation on blastn that is slower but more sensitive. It will find more dissimilar matches so it is ideal for cross-species comparison.
- **blastp**: Compares an amino acid query sequence against a protein sequence database.
- **blastx**: Compares a nucleotide query sequence translated in all reading frames against a protein sequence database. You could use this option to find potential translation products of an unknown nucleotide sequence.
- **tblastn**: Compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames.
- **tblastx**: Compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database. Please note that the tblastx program cannot be used with the nr database on the BLAST Web page because it is too computationally intensive.

Geneious is able to run NCBI BLAST on many different databases. Some of these databases are non-redundant in order to reduce duplicate hits. The databases that can be searched are shown in the following tables.

You can quickly and easily BLAST against any of these databases using any of the available BLAST programs via the Sequence Search operation. This operation can be accessed by going to the Tools menu or by right-clicking (Ctrl+click on Mac OS X) on a sequence document and choosing "Sequence Search". This will bring up the sequence search options.

Geneious gives you the option of searching against a database using either your currently selected sequence documents or a sequence you enter manually. If you choose to enter your sequence manually, then Geneious will display a large text box in which you can enter your query sequence as either unformatted text or FASTA format.

Select your database using the first drop-down box. Databases are grouped together under their respective services. The available programs in the second drop-down box will depend on the database you have chosen.

Geneious also allows you to specify most of the advanced options that are available in BLAST. To access the advanced options click the "More Options" button which is in the bottom left

Table 2.2: Nucleotide sequence searches in the BLAST databases

Database	Nucleotide searches
nr	All non-redundant GenBank+EMBL+DDBJ+PDB sequences(no EST, STS, GSS or HTGS sequences)
genome	Genomic entries from NCBI's Reference Sequence project
est	Database of GenBank + EMBL + DDBJ sequences from EST Divisions
est_human	Human subset of est
est_mouse	Mouse subset of est
est_others	Non-Human, non-mouse subset of est
gss	Genome Survey Sequence, includes single-pass genomic data, exon-trapped sequences, and Alu PCR sequences.
htgs	Unfinished High Throughput Genomic Sequences: phases 0, 1 and 2 (finished, phase 3 HTG sequences are in nr)
pat	Nucleotide sequences derived from the Patent division of GenBank
PDB	Sequences derived from the 3D-structures of proteins from PDB
month	All new/updated GenBank+EMBL+DDBJ+PDB sequences released in the last 30 days.
RefSeq	NCBI-curated, non-redundant sets of sequences.
dbsts	Database of GenBank+EMBL+DDBJ sequences from STS Divisions
chromosome	A database with complete genomes and chromosomes from the NCBI Reference Sequence project.
wgs	A database for whole genome shotgun sequence entries.
env_nt	This contains DNA sequences from the environment, i.e all organisms put together

Table 2.3: Protein sequence searches in the BLAST databases

Database	Protein searches
env_nr	Translations of sequences in env_nt
month	All new/updated GenBank coding region (CDS) translations +PDB+SwissProt+PIR released in last 30 days
nr	All non-redundant GenBank coding region (CDS) translations+PDB+SwissProt+PIR+PRF
pat	Protein sequences derived from the Patent division of GenBank
PDB	Sequences derived from 3D structure Brookhaven PDB
RefSeq	RefSeq protein sequences from NCBI's Reference Sequence Project
SwissProt	Curated protein sequences information from EMBL

of the Sequence Search options. The available options vary depending on the kind of BLAST search you have selected. For details on each of the options you can hover your mouse over the option to see a short description or refer to the NCBI BLAST documentation at http://www.ncbi.nlm.nih.gov/blast/blastcgihelp.shtml.

Once a search has started, a results folder will be created under the Searches folder in the Sources panel. Search progress is shown in the document table. The search can be cancelled by clicking on the red square labelled "Stop" (See Figure 2.8).

When using the "Standard" search type, each search hit is displayed separately in the document table sorted by "E value". As well as standard columns like "Name", search hits can also be sorted by "Coverage" and a special "Grade" column which is calculated by Geneious. The "Grade" column is a percentage calculated by combining the query coverage, e-value and identity values for each hit with weights 0.5, 0.25 and 0.25 respectively. This allows you to sort hits such that the longest, highest identity hits are at the top.

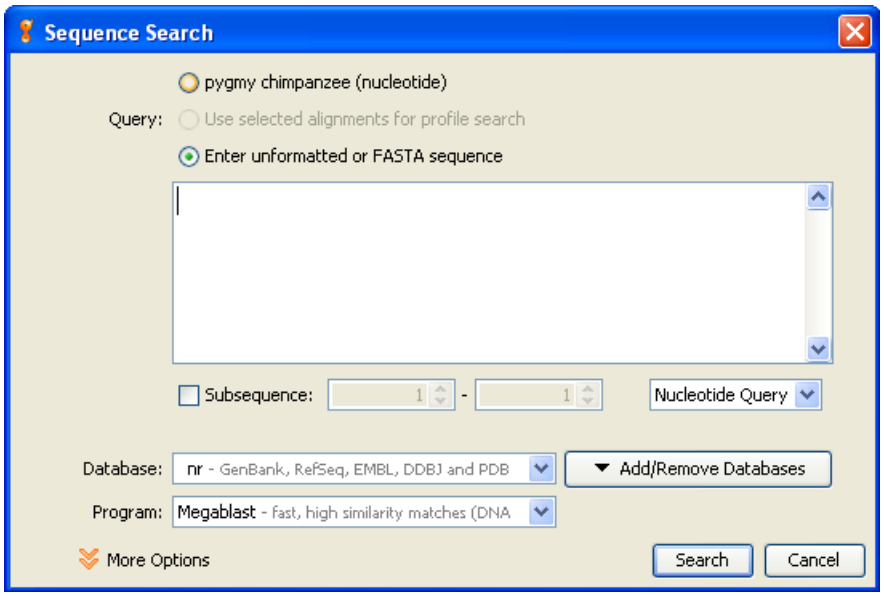


Figure 2.7: Sequence Search Options

Once the search has completed the results can be moved to your local database at your convenience. If your query sequence was annotated then any annotations that cover the hit region will be transfered to the BLAST hit document.

You can also download the full database sequence that corresponds to a BLAST hit. To retrieve the full sequence select a BLAST alignment and go to "File"→"Download Documents" or click the **Download Full Sequence(s)** button located above the viewer tabs. The full sequence will be available in the "Sequence View" tab once the download has completed. In addition the annotations from the full sequence will be transfered over to the BLAST alignment (see Figure 2.10).

If you have a mirror of the NCBI BLAST databases you can set Geneious to use this by going to "Tools"→"Add/Remove Databases"→"Set Up Search Services". This will bring up a dialog that allows you to change the setup for various search services in Geneious. Choose NCBI using the service drop-down box at the top of the dialog. Enter the URL for the mirror and click 'OK' to apply the new settings. You can also edit the databases that show up in Geneious by clicking on Edit Databases. This will only change the databases that Geneious displays and will not have any effect on the actual databases on the BLAST server.

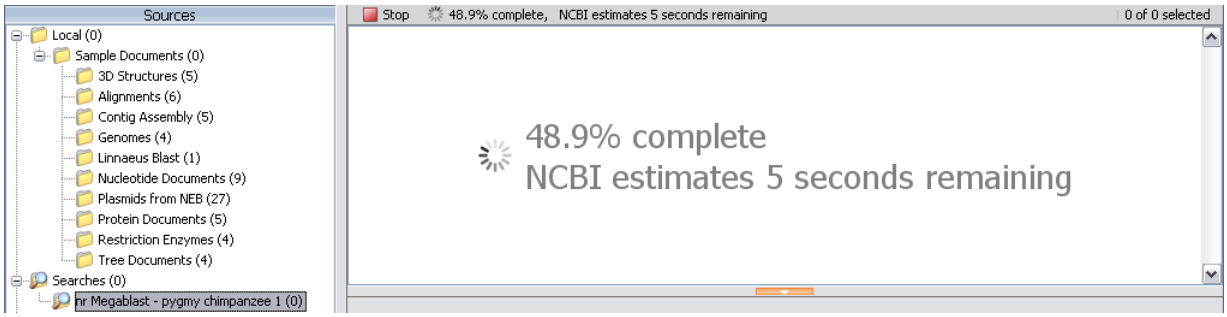


Figure 2.8: Sequence Search in Progress

2.5 Storing data - Your Local Documents

Geneious can be used to store your documents locally. Under the "Local" folder in the Services Panel you are able to create sub-folders to organize and store a variety of document types (Table 2.4).

This is also where you can set up special folders to receive documents that are downloaded by a Geneious agent. To create a new folder in Geneious, select the "Local" folder or a sub-folder icon in the services panel and right-click (Ctrl+click on Mac OS X). This will pop up a menu. Clicking on "New folder..." opens a dialog that will prompt you to name the folder. The named folder is then created as a sub-folder of the folder that you originally right-clicked on.

Important. Search results will be lost when you exit Geneious unless the downloaded documents have been copied or moved to one of your local folders.

In Geneious you can create new folders, rename existing folders, delete and export folders. All these choices are available by either right-clicking on the folder, clicking on the action menu (Mac OS X), or by holding down the Ctrl button and clicking (Mac OS X). Also in Mac OS X, you can also use the plus (+) and minus (-) buttons located at the bottom of the service panel to create and delete folders.

2.5.1 Transferring data

It is quick and easy to transfer data to your local folders from either a Geneious database search or from your computer's hard drive. Please check you have already set up your destination folders before continuing.

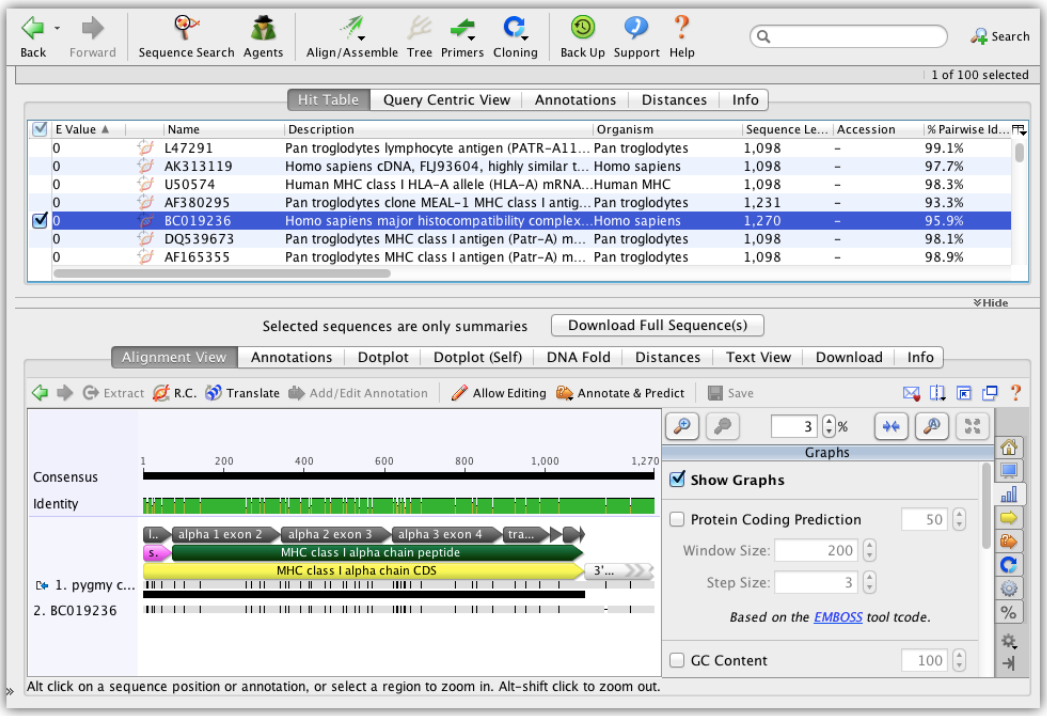


Figure 2.9: Sequence Search Complete

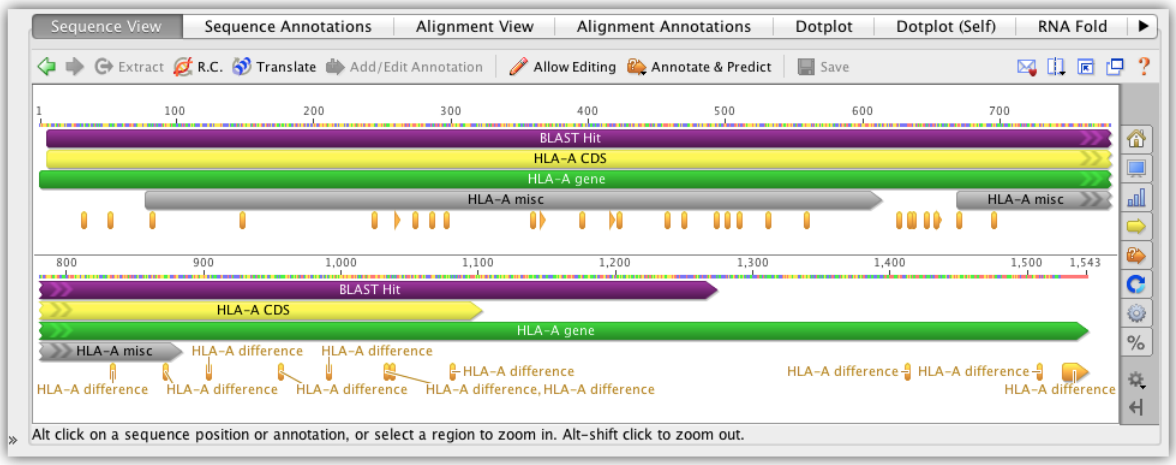
Moving documents from Geneious searches to your Local folders

There are a number of ways to do this.

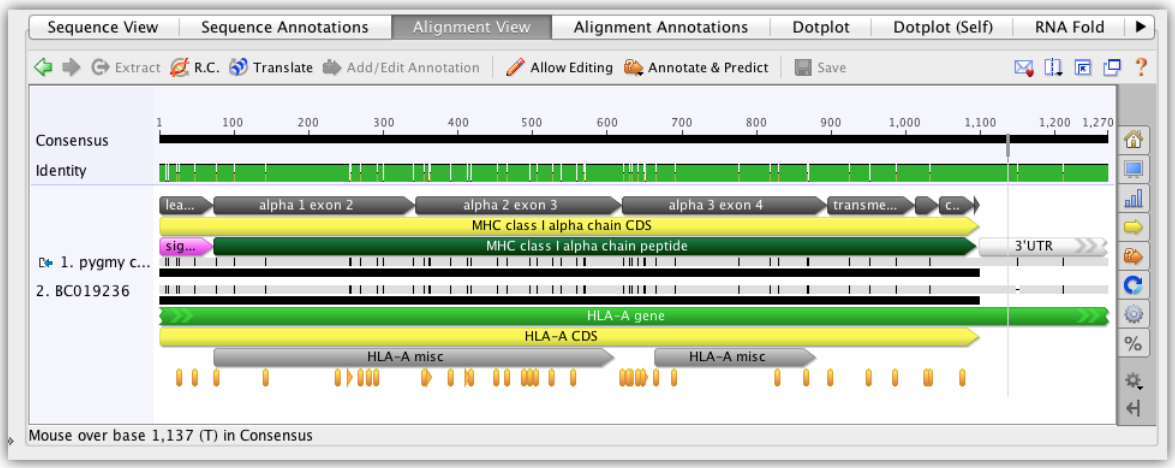
Drag and drop. This is quickest and easiest. Select the documents that you want to move. Then, while holding the mouse button down, drag them over to the desired folder and release. If you dragged documents from one local folder to another, this action will move the documents – so that a copy of the document is not left in the original location. In external databases such as NCBI the documents will be copied, leaving one in its original location.

Drag and copy. While dragging a document over to your folder, hold the Ctrl key (Alt/Option key on Mac OS X) down. This places a copy of the document in the target folder while leaving a copy in the original location. This is useful if you want copies in different folders. Folders themselves can also be dragged and dropped to move them but they cannot be copied.

The Edit menu. Select the document and then open the Edit menu on the menu bar. Click on "Cut" (Ctrl+X/⌘+X), or "Copy" (Ctrl+C/⌘+C). Select the destination folder and "Paste" (Ctrl+V/⌘+V) the document into it.



(a) Complete Database Sequence



(b) Annotations Transfered to Alignment

Figure 2.10: Document After Full Sequence Download

Table 2.4: Geneious document types

Document type	Geneious Icon
Nucleotide sequence	
Oligo sequences	
Enzyme Sets	
Chromatogram	
Contig	
Protein sequence	
Pfam domain sequence	
Phylogenetic tree	
3D structure	
Sequence alignment	
Journal articles	
PDF	
Other documents	

2.5.2 Deleting Data and "Deleted Items"

When a folder or document is deleted, Geneious moves the data to the "Deleted Items" folder instead of erasing it immediately. This means the data can be recovered if it was deleted by mistake. Pressing the Delete key is the easiest way to move the selected folder or documents to the "Deleted Items" folder.

To recover documents or folders from "Deleted Items" you can either move them manually to another location or use "Restore from Deleted Items" ("Put Back from Deleted Items" on Mac OS) in the File menu to automatically move them to folder they were deleted from.

The "Deleted Items" folder should be cleared periodically to keep hard drive space free. This can be done by selecting "Erase All Deleted Items" from the File menu. Geneious will warn you if "Deleted Items" contains a large amount of data.

To erase a document immediately without moving it to "Deleted Items", use "Erase Document Immediately" in the File menu (or press Shift+Delete).

Many of these actions can also be accessed by right clicking on a folder or document.

2.5.3 Document History

When a document is created or modified information regarding this change is also saved. This data can be viewed in the History Viewer, described in section 3.8. Saving document history can be disabled for performance or privacy reasons by going to the Appearance and Behaviour tab in Preferences, see section 2.9.

2.5.4 Searching your Local folders

The "Services Panel" allows you to browse your Local folder hierarchy. Next to each folder name in the hierarchy is the number of documents it contains in brackets. When the Local folder or a sub-folder is collapsed (minimized), the brackets next to the folder shows how many files are contained in that folder as well as all of its sub-folders. In addition, if some of the documents in a folder are unread, the number of unread documents will also appear in the brackets.

You can search the Local folder (and sub-folders) the same way you search the public databases by clicking on the "Search" icon. If you have defined a new type of meta-data in Geneious, and that meta-data has been added to a document, it will also be added to the "Advanced Search" criteria. Look at an example of a new meta-data type called "Protein size", which takes a text value for the protein in kDa (kiloDaltons) (see Figure 2.11).

Important: You must use quotation marks ("") if "!", "@", "$", and blank spaces (" ") are part of your search criteria. No quotation marks lead to unreliable results.

Wild card searches

When you are looking for all matches to a partial word, use the asterisk (*). For example, typing "oxi*" would return matches such as oxidase, oxidation, oxido-reductase, and oxide. This is useful for performing generic searches. You can also place asterisks (*) in the middle of the word or at the beginning. This feature is available only for local documents.

Similarity ("BLAST-like") searching

It is possible to search your local documents not only for text occurrences but by similarity to sequence fragments. Click the small arrow at the bottom of the large T to the left of the

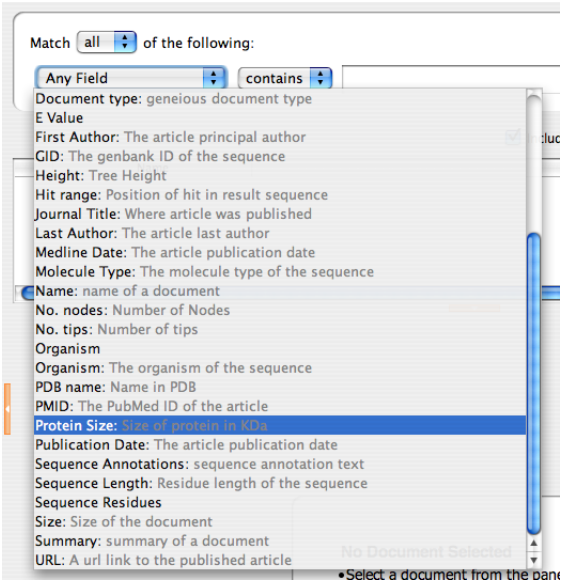

Match all of the following:
Any Field
contains
Document type: geneious document type
E Value
First Author: The article principal author
GID: The genbank ID of the sequence
Height: Tree Height
Hit range: Position of hit in result sequence
Journal Title: Where article was published
Last Author: The article last author
Medline Date: The article publication date
Molecule Type: The molecule type of the sequence
Name: name of a document
No. nodes: Number of Nodes
No. tips: Number of tips
Organism
Organism: The organism of the sequence
PDB name: Name in PDB
PMID: The PubMed ID of the article
Protein Size: Size of protein in KDa
Publication Date: The article publication date
Sequence Annotations: sequence annotation text
Sequence Length: Residue length of the sequence
Sequence Residues
Size: Size of the document
Summary: summary of a document
URL: A url link to the published article
No Document Selected
•Select a document from the ban

Figure 2.11: Searching the local documents on a user-defined field

search dialog, select "Nucleotide similarity search" or "Protein similarity search" and enter the sequence text. Geneious will try to guess the type of search based on the text, so that simply entering or pasting a sequence fragment may change the search type automatically.

The search locates documents containing a similar string of residues, and orders them in decreasing order of similarity to the string. The ordering is based on calculating an E-value for each match. You can read more about the E-value in subsection 2.4.4.

For the search to be successful, you need to specify a minimum of 11 nucleotides and 3 amino acids. Note that search times depend on the number and size of your sequence documents, and so may take a long time to complete.

2.5.5 Checking and changing the location of your Local folders

To check where your Local folders are being stored on your hard drive, open the Tools menu in the Menu Bar. Click "Tools" → "Preferences" → "General". Your documents are stored at the location specified by the "Data Storage Location" field (see Figure 2.12). You can change this location by clicking the "Browse" button and selecting a new location. Geneious will remember this new location when you exit.

Warning: Do not place your local database on a network share, or use a synchronization tool such as DropBox. Geneious accesses the local database frequently so performance will be very poor and your data will get corrupted.

2.5.6 Find Duplicates

"Find Duplicates" is located under the "Edit" menu and is used to identify sequences and other documents that are duplicated. It can check for duplicates within a selected set of documents, all documents in a folder or in the sequences of a single alignment or sequence list. Duplicates can be identified by database ID (e.g. accession) or by the residues/bases.

Once run, the operation will select all but one copy of a duplicated document. This means they can be deleted or easily moved to another folder, leaving one copy behind.

If you are searching for duplicates within sequences of a single alignment or sequence list, you also have the option to extract unique sequences from the list.

2.5.7 Batch Rename

"Batch rename" is located under the "Edit" menu and is used to edit the names of many documents in one step. It has options to replace the names with a combination of values from other columns (e.g. organism or accession). It can also add fixed text to the beginning or end of each name.

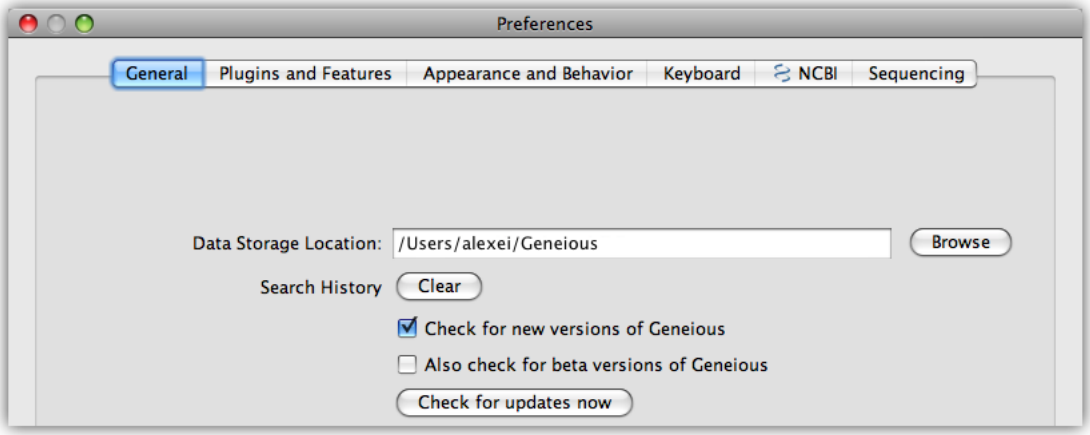


Figure 2.12: Setting the location of your local documents

2.6 Agents

Geneious offers a simple way for you to continuously receive the latest information on genomes, sequences, and protein structures. This feature is called an agent. Each agent is a user-defined,

automated search. You can instruct an agent to search any Geneious accessible database at regular intervals (e.g. weekly) including your contacts on Collaboration. This simple but powerful feature ensures that you never miss that critical article or DNA sequence. To manage agents click on the agent icon in the toolbar. An agent has to be set up before it can be used.

2.6.1 Creating agents

To set up an Agent click the Agents icon and the create button. You now need to specify a set of search criteria in the exact same way as you do for search, the database to search, search frequency and the folder you wish the agent to deliver its results to.

The search frequency may be specified in minutes, hours, days or weeks. You can only use whole numbers.

Selecting "Only get documents created after today" will cause the agent to check what documents are currently available when the agent is created. Then when the agent searches it will only get documents that are new since it was created, e.g. If you have already read all publications by a particular author and you want the agent to only get publications released in the future.

Alternatively you can click the "Create Agent..." button which is available in some advanced search panels. This will use the advanced search options you have entered to create the agent.

The easiest way to organize your search results is to create a new folder and name it appropriately. You can do that by navigating to the parent folder in the "Deliver to" box and click "New Folder", or by creating a new folder beforehand,

1. Right-click (Ctrl+click on Mac OS X) on the "Sample Documents" or "Local" folders. This brings up a popup menu with a "New Folder..." option.
2. Create a new folder and name it according to the contents of the search. (For example, type "CytB" if searching for cytochrome b complex.)
3. Once created, select the new folder. You can now select the "Create" or "Create and Run". The agent will then be added to the list in the agent dialog and it will perform its first search if you clicked "Create and Run". Otherwise it will wait until its next scheduled search.

2.6.2 Checking agents

Once you have created one or more agents, Geneious allows you to quickly view their status in the agents window which is accessible from the toolbar. Your agents' details are presented in several columns: *Enable*, *Action*, *Status* and *Deliver To*.

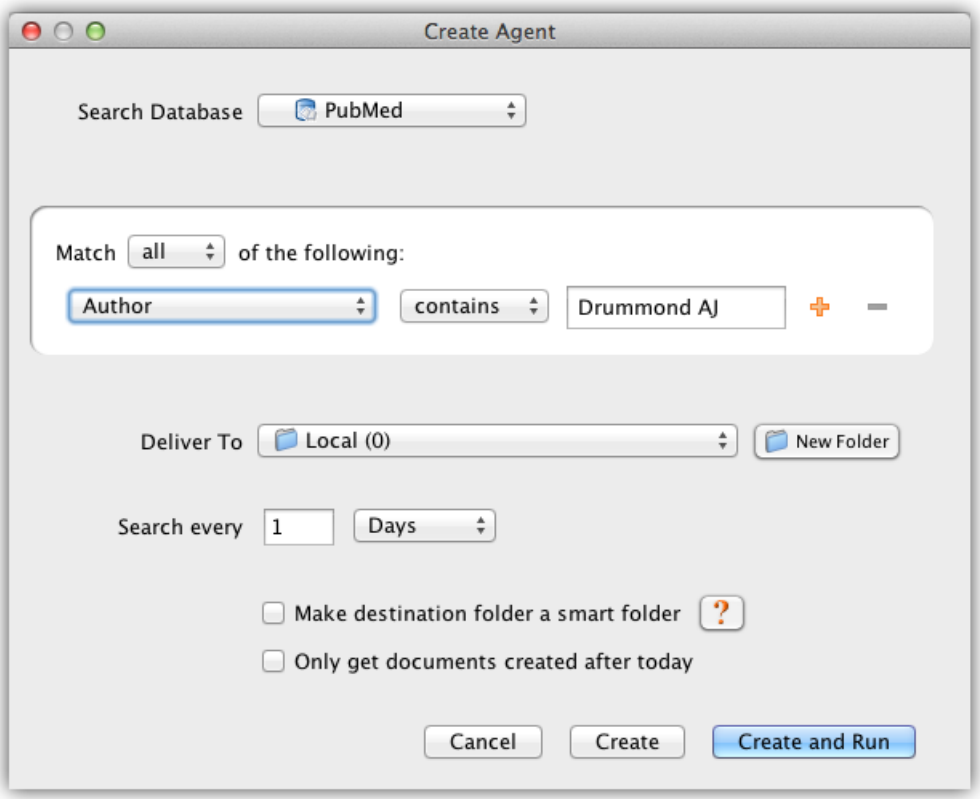


Figure 2.13: The Create Agent Dialog

Enable This column contains a check box showing whether the agent is enabled. *Action*. This summarizes the user-defined search criteria. It contains:

1. Details of the database accessed. For example, Nucleotide and Genome under NCBI.
2. The search type the Agent performed, e.g. "keyword".
3. The words the user entered in the search field for the Agent to match against.

Status. This indicates what the Agent is currently doing. The status will be one of the following:

- "Next search in x time" e.g. 18 hours. The agent is waiting until its next scheduled search and it will search when this time is reached.
- "Searching." These are shown in bold. The agent is currently searching.
- "Disabled." The agent will not perform any searches.
- "Service unavailable." The agent cannot find the database it is scheduled to search. This will happen if the database plugin has been uninstalled or if for example the Collaboration contact is offline currently.
- "No search scheduled" The agent is enabled but doesn't have a search scheduled. To correct this click the "Run now" button in the agent dialog to have it search immediately and schedule a new search.

Deliver To. This names the destination folder for the downloaded documents. This is usually your Local Documents or one of your local folders.

Note. If you close Geneious while an agent is running, it will stop in mid-search. It will resume searching when Geneious is restarted. Also, all downloaded files are stored in the destination folder and are marked "unread" until viewed for the first time.

2.6.3 Manipulating an agent

Once an agent has been set up, it can be disabled, enabled, edited, deleted and run. All these options are available from within the Agents dialog.

- *Enable or disable* an agent by clicking the check box in the Enable column.
- "Run Now" Cause the agent to search immediately
- "Cancel" If the agent is currently searching this can be clicked to stop the search.

- "Edit" Click this to change an agent's database, search criteria, destination or search interval.
- "Delete" Delete the agent permanently. Any documents retrieved by the agent will remain in your local documents.

2.7 Filtering and Similarity sorting

The "Filter" allows you to instantly identify documents in the document table matching chosen keywords. It is located in the top right hand corner of the Main Toolbar.

Type in the text you are searching for and Geneious will display all the documents that match this text and hide all other documents in the Document Table. To view all the documents in a folder, clear the Filter box of text or click the button.

The "Sort" button in the toolbar provides two actions in a popup menu. Sort by similarity is available when a single sequence document is selected in the Document Table. It will rank all other sequences by their similarity to the selected sequence. The most similar sequence is placed at the top and the least similar sequence at the bottom. This also produces an E-value column describing how similar the sequences are to the selected one. The "Remove Sort by Similarity" action will remove the E-value column and return the table to its previous sorting.

2.7.1 Filtering on-the-fly

Filtering can be used while searching for documents via public databases, filtering data as it is being downloaded. Type in the appropriate text in the Filter Box and only those documents that match both the original criteria (as specified by the search terms) and the "Filter" text will be displayed. This is an effective way of filtering within your search results.

2.8 Meta-Data

Meta-data allow you to add arbitrary information to any of your local documents, and any meta-data that you add can be treated as user-defined fields for use in sorting, searching and filtering your documents.

Where can I add Meta-Data?

You can add meta-data to any of your local documents, including molecular sequences, phylogenetic trees and journal articles. You cannot add meta-data to search results from NCBI or

EMBL etc until the documents are copied into one of your local folders.

The Properties View

All documents have an "Info" tab in the document viewer panel which contains a "Properties" tab. This is where standard properties of documents such as name and description are displayed along with any meta-data. To add meta-data to your document, select the "Add a Meta-Data" button on the toolbar and then choose from the available types. Selecting a meta-data type will create an empty instance of that type. To fill meta-data values just start typing into the fields.

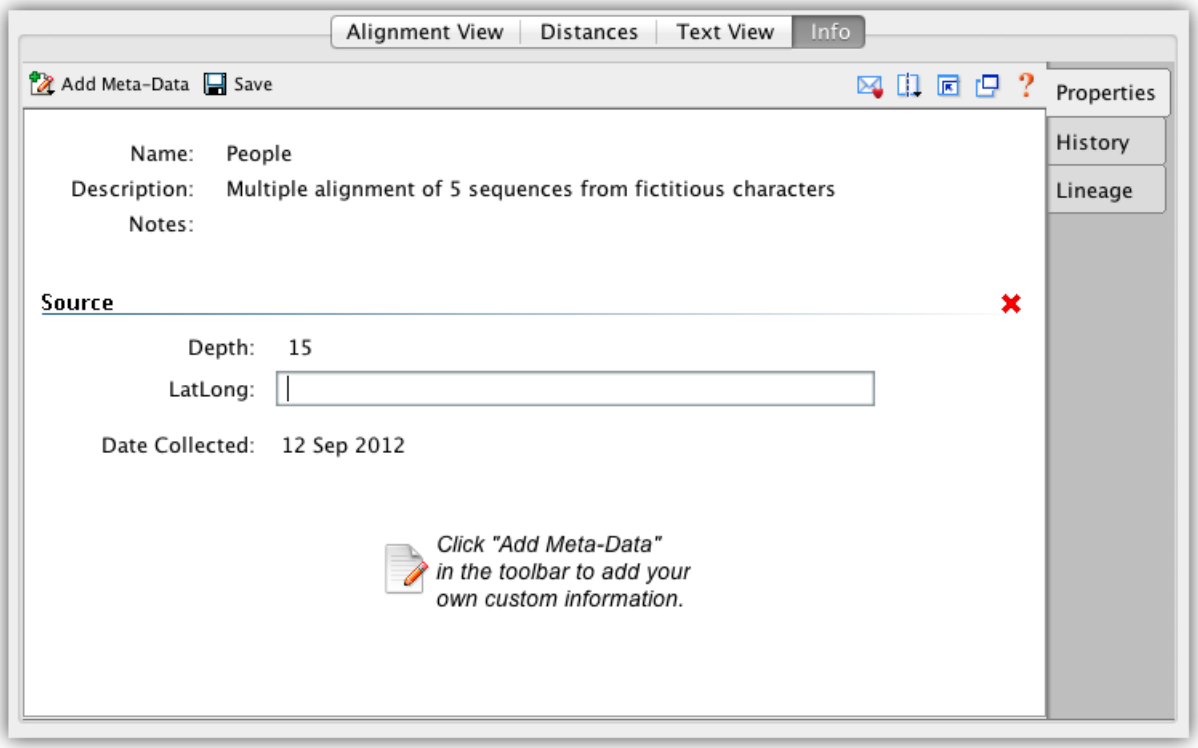


Figure 2.14: The Properties View

2.8.1 Editing Meta-Data

To edit meta-data fields, simply click on the field and enter your data. Some fields may have constraints (which you can edit in the Edit Meta-Data Types dialog, *(see 2.8.2)*. If the data you

have entered does not conform to the constraints of the field, it will be displayed in red and you will be shown the field's constraints in a tooltip.

***Tip: To enter a new line in a text field, press* Shift+enter *or* Ctrl+enter**

When multiple documents are selected, the Properties view displays all of the fields and meta-data belonging to the selected documents. When all documents have the same value for a field, it is displayed in the viewer. If the documents have different values, or some of the selected documents do not have a value, then the field will show that it represents multiple values. Changes made to the fields will apply to all selected documents.

2.8.2 Editing Meta-Data Types

To edit meta-data types, click the "Add Meta-Data" button on the viewer toolbar and select "Edit meta-data types...". This will bring up a window similar to that displayed in figure 2.16.

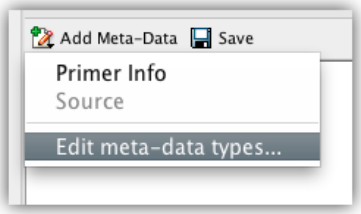


Figure 2.15: Edit Meta-Data Types

Creating Meta-Data Types

Geneious does not restrict you to the meta-data types that it comes with. You can create your own types to store any information you want.

To create a new type, click on the Create button in the left-hand panel of the Edit Meta-Data Types window. This creates a new type, with one empty field, and displays it in the panel to the right.

Note. The "Name" and "Description" fields distinguish your meta-data type from other user-defined types. They do not have any constraints.

Next, you need to decide what values your Meta-Data Type will store by specifying its fields:

Field name. This defines what the field will be called. It will be displayed alongside columns such as Description and Creation Date in the Documents Table. You can have more than one Field in a single Meta-Data Type - to add or remove a field from the type, click the + or - buttons to the right of the field.

Field type. This describes the kind of information that the column contains such as Text, Integer, and True/False. The full list of choices in Geneious is shown in figure 2.16.

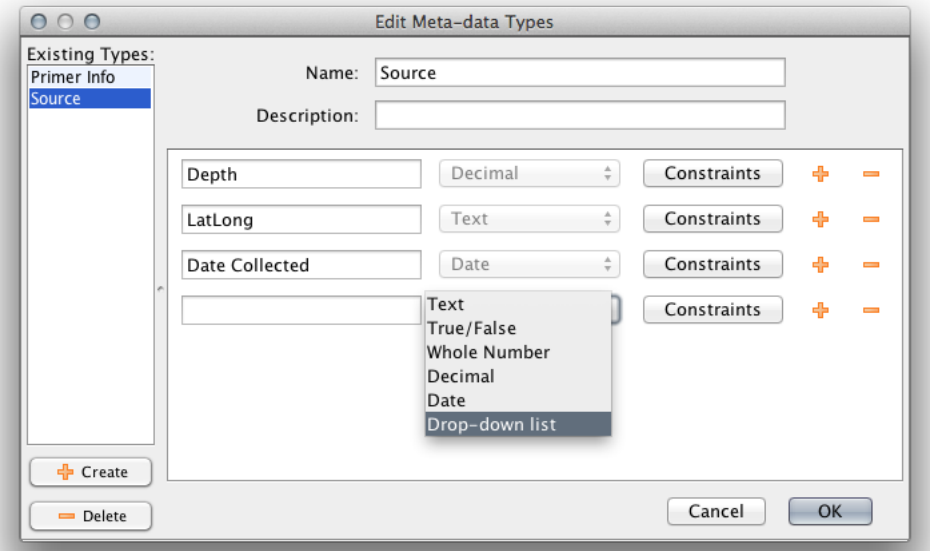


Figure 2.16: The Edit Meta-Data Types window

Constraints. These are limiting factors on the data and are specific to each field type. For example, numbers have numerical constraints – is greater than, is less than, is greater or equal to, and is less or equal to. These can be changed to suit. The constraints for each field can be viewed by clicking the "View Constraints" button next to the field. This will show a pop-up menu with the constraints you have chosen. *(see figure 2.17)*

Using Meta-Data

The main purpose of meta-data is to add user defined information to Geneious documents. However, meta-data can be searched for and filtered as well. Also, documents can be sorted according to meta-data values.

Searching - Once meta-data is added to a document, it is automatically added to the standard search fields. These are listed under the "Advanced Search" options in the Document Table. From then on, you can use them to search your Local Documents. If you have more than one Field in a meta-data type, they will all appear as searchable fields in the search criteria.

Filtering - Meta-data values can be used to filter the documents being viewed. To do so, type a value into the "Filter Box" in the right hand side of the Toolbar. Only matching documents will be shown.

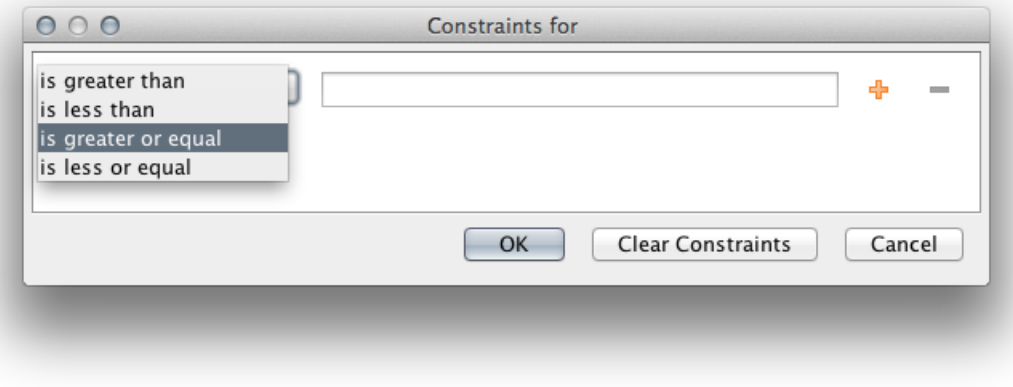


Figure 2.17: The Edit Constraints window

Sorting - Any meta-data fields added to documents will also appear as columns in the Document Table. These new columns can be used to order the table.

2.9 Preferences

You can access the preferences screen in two ways:

1. Shortcut keys: Ctrl+Shift+P (Windows/Linux), ⌘+Shift+P (Mac OS X)
2. Select the Tools Menu and click Preferences.

There are several sections in the preferences window which are presented as tabs. The most important of these are described below.

2.9.1 General

This contains connection settings, data storage details for your local documents, automatic new version checking and a "Search History".

"Check for new versions of Geneious" Enable this to have Geneious check for the release of new versions everytime it is started. If a new version has been released Geneious will tell you and give you a link to download it.

"Also check for beta versions of Geneious" Enable this to also have Geneious alert you when new beta versions are released. A beta version is a version that is released before the official release for the purposes of testing. It may therefore be less stable than official releases.

"Max memory available to Geneious" allows you to enter how many megabytes of your computers memory (RAM) you wish to allow Geneious to use. Specifically this sets the maximum Java heap size. You should never set this to be the total memory of your computer as you need to leave some RAM available for your operating system. For example, if you have 4GB available, you should set Geneious to have no more than 2GB so the operating system will have enough room to perform its tasks. Even on machines with a lot more memory, it is still a good idea to leave 2GB or more for the operating system to keep your computer running smoothly.

Connection settings. These are described in the troubleshooting section of the manual.

2.9.2 Plugins and Features

The "Plugins and Features" tab (Figure 2.18) lets you manage downloadable plugins and change the features available in Geneious.

- **Available Plugins:** Lists all plugins which are currently available for download from the Geneious website which aren't already installed. Each plugin is listed with a status which can be a star (for exciting plugins), New or Beta. Click the Info button to read more about the plugin or click the Install button to download the plugin and install it.
- **Installed Plugins:** Lists all plugins you currently have installed. Click the uninstall button next to a plugin to remove it.
- **Install plugin from a gplugin file:** If you have downloaded a plugin from our website or obtained one from another source (usually in .gplugin format) you can install it by clicking this button or by dragging the plugin file in to Geneious.
- **Check for plugin updates now:** Checks if there are any new versions available for the plugins you have installed.
- **Automatically check for updates to installed plugins:** If checked, Geneious will check for new versions of your installed plugins each time the program is started.
- **Tell me when new plugins are released:** Changes the way the program notifies you about new plugin releases.
- **Also check for beta releases of plugins:** Plugins are sometimes initially released as a beta for the purposes of testing before the officially release. Check this to be notified about the release of beta plugins.
- **Customize feature set:** Click this to see a list of all features in Geneious. Any number of these can be turned off by un-checking the Enabled box next to each feature. You might like to turn of the Tree Builder and Tree Viewer plugins if you don't do phylogenetics for example.

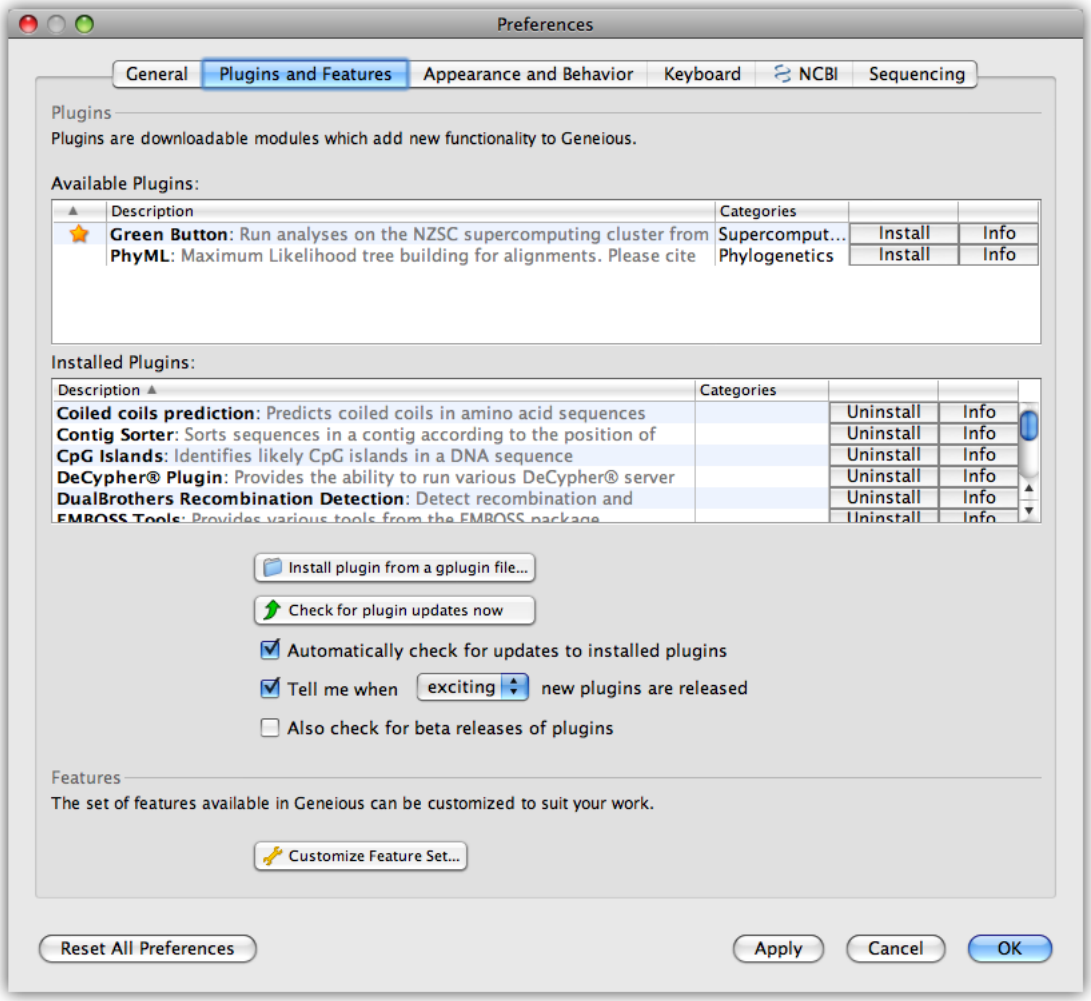


Figure 2.18: The plugins preferences in Geneious

2.9.3 Appearance and Behavior

Here you can change the way Geneious looks and the way it interacts with you.

Appearance options allow you to change the way the main toolbar and the document table look.

Behaviour options allow you to change the way newly created documents are handled. Such as whether they are selected straight away and where they should be saved to.

2.9.4 Keyboard

This section contains a list of Geneious functions and allows you to define keyboard shortcuts to them. Shortcuts that are already defined are highlighted in blue.

Setting shortcuts can help you quickly navigate through Geneious without using the mouse and also allows you to redefine shortcuts to ones you may be familiar with from other programs.

Double click on a function to bring up a window to enter your new keyboard shortcut. If you use one that is already assigned, Geneious will tell you what function currently has that shortcut.

2.9.5 Sequencing

This tab has options for the management of trace files and assemblies:

- **Confidence:** Set the threshold values of base call confidence used to determine if a base call is low, medium or high quality. This affects the binning parameters described below as well as the Confidence color scheme in the Sequence View.
- **Sequence binning options:** Specifies the requirements for individual traces to be binned as medium or high quality overall. To see the Bin for a trace, turn on the Bin column under Table Columns in the View menu.
- **Assembly binning options:** Specifies the requirements for assembly documents to be binned as medium or high quality overall. To see the Bin for an assembly, turn on the Bin column under Table Columns in the View menu.
- **Track binning history in meta-data:** When turned on, meta-data will be added to traces when they are trimmed (see the Properties view tab). This meta-data will then updated every time the trace is re-trimmed, maintaining a history of the trimming.

- **Enable per folder/document binning:** When turned on, the **Set Binning Parameters** option is added to the Sequence menu. This allows you to select an individual folder or set of documents and set the binning parameters to use on those documents instead of the global ones set in the Preferences.

2.10 Printing and Saving Images

Geneious allows you to print (or save as an image) the current display for any document viewer. This includes the sequence viewer, tree view, dotplot, and text view.

2.10.1 Printing

Choose "print" from the file menu. The following options are available

Portrait or landscape. Controls the orientation of the page.

Scale. Can be used to decrease or increase the size of everything in the view, while still printing within the same region of the page. For many types of document views, this will cause it to wrap to the following line earlier, usually requiring more pages.

Size. Controls the size the printed region on the paper. Effectively, increasing the size, reduces the margins on the page.

2.10.2 Saving Images

Choose "save to image file" from the file menu. The following options are available

Size. Controls the size of the image to be saved. Depending on the document view being saved, these may be fixed or configurable. For example, with the sequence viewer, if wrapping is on, you are able to choose the width at which the sequence is wrapped, but if wrapping is off, both the width and height will be fixed.

Format. Controls image format. Vector formats (PDF, SVG and EMF) are ideal for publication because they won't become pixelated. Raster formats (PNG and JPG) are easier to share, great for emailing posting on the web. If you plan to use the image in Microsoft Office then EMF format is recommended. Microsoft Office for Mac can't ungroup EMF files like the Windows version can unfortunately. LibreOffice for Mac, Windows or Linux can and allows you to edit the individual elements.

Resolution. Only applies to raster formats (PNG and JPG) and is used to increase the number of pixels in the saved image.

2.11 Back up

It is important to keep frequent back ups of your data because computers can fail suddenly and unexpectedly. A computer can be replaced, but your data is much harder to replace. The best way to back up all of your data and settings in Geneious is to use the *Back Up* button in the toolbar or select *Back Up Data* in the File menu.

Backing up your data directory manually is not recommended because the Geneious database structure is complex and many programs will fail to back it up properly.

The back up command has two options:

- **Export selected folder:** This will export the selected folder (including all subfolders) to a Geneious format file. This allows you to back up an individual project within your database. The backup can also be imported in to an existing database by drag and drop. If you have finished working on a project it is a good idea to back it up in this way then delete it from inside Geneious to keep the size of your database down and improve the performance of Geneious. You should keep archive backups in addition to these because this backup will miss your settings and data outside the selected folder.
- **Archive all data and settings:** This is equivalent to creating a zip archive of your entire Geneious data directory which includes all your data, preferences, searches and agents. This type of backup cannot be directly imported in to an existing database, when it is loaded everything in Geneious will revert to how it was when you took the backup.

2.11.1 Restoring a backup

- **Geneious format backup:** The easiest way to restore this is to drag and drop the Geneious file in to the folder in Geneious where you want it to go. Alternatively you can use *Restore Backup* in the File menu and the backup will be added under the *Local* folder in your current database.
- **Archive all data and settings:** It is strongly recommended that you use *Restore Backup* in the file menu to load the zip file rather than unzipping it manually. Some operating systems may not be able to unzip the data correctly. The *Restore Backup* command will unzip your backed up data directory to a folder of your choosing which you can then load immediately. If you choose not to load it immediately you can switch to the restored data directory by going to *Preferences* in the Tools menu and changing the *Data Storage Location* on the General tab.

Chapter 3

Document Viewers

3.1 General viewer controls

There are several general options which are available on all viewers. These can be accessed through the ”View” menu or on the right hand side of the toolbar above the viewer.

Split View: Provides several options for splitting the view so that multiple views are shown simultaneously for one document. When the view is split, selection of annotations and regions of the sequence are synchronized across the viewers. To close split views click the button which is also on the right of the toolbar.

Expand View: Expands the document view panel to fill the main window by hiding the sources panel on the right and the document table above. Clicking this again will return the layout to it's original state.

New Window: Opens another view of the current document in a separate window. This allows you to have several documents open at once and gives more space for viewing. This can also be achieved by double clicking in the document table.

3.2 The Sequence (and alignment) Viewer

The “Sequence view” tab in the Document Viewer panel is available for Nucleotide sequences, Protein sequences, Alignments and 3D structure documents. If an alignment is selected, this will be called “Alignment View” or “Contig View” if a contig is selected. The options available vary with the kind of sequence data being viewed.

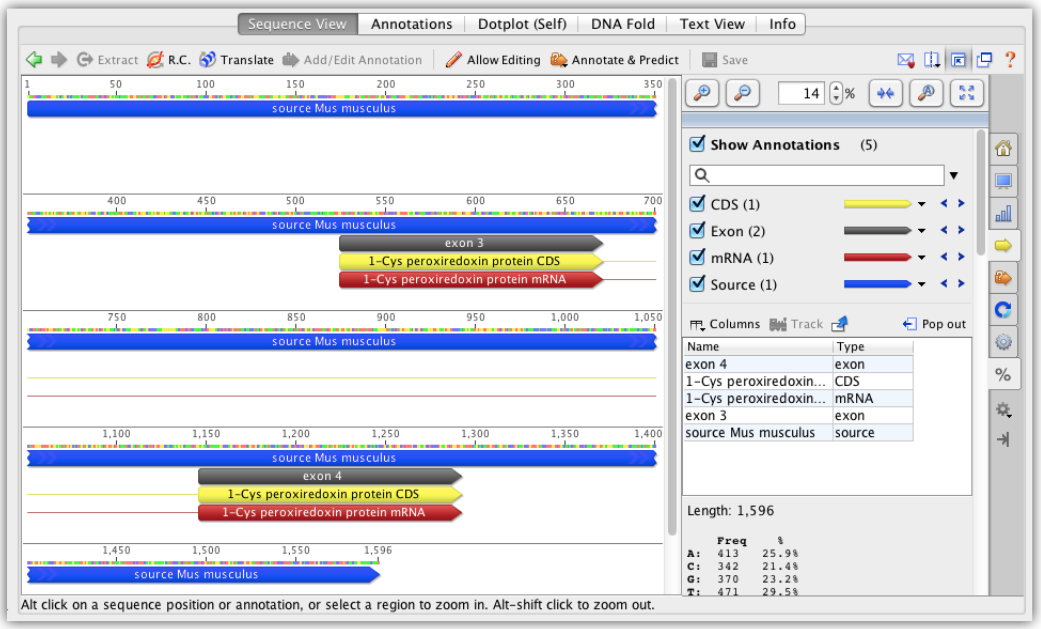


Figure 3.1: A view of an annotated nucleotide sequence in Geneious

3.2.1 Zoom level

The plus and minus buttons increase and decrease the magnification of the sequence by 50%, or by 30% if the magnification is already above 50%.

zooms in to fit the selected region in the available viewing area.

zooms to 100%. The 100% zoom level allows for comfortable reading of the sequence.

zooms out so as to fit the entire sequence in the available viewing area.

Zooming can also be quickly achieved by holding down the zoom modifier key which is the Ctrl key on Windows/Linux or the Alt/Option key on Mac OS X and clicking. When the zoom key is pressed a magnifying glass mouse cursor will be displayed.

- Hold the zoom key and left click on the sequence to zoom in.
- Hold the zoom key and Shift key to zoom out.
- Hold the zoom key and turn the scroll wheel on your mouse (if you have one) to zoom in and out.
- Hold the zoom key and click on an annotation to zoom to that annotation

You can also pan in the Sequence View by holding Ctrl+Alt (⌘+Alt on Mac OS X) and clicking on the sequence and dragging.

3.2.2 Circular View

When a circular sequence is selected, the default view is to display the sequence as circular. The view can be rotated by using the scrollbar at the bottom or by turning the mouse wheel. Even though a sequence is circular, you can display it as a linear sequence using the "Linear view on circular sequence" checkbox under the "Layout" section of Advanced.

3.2.3 Genome View

The genome view (Figure 3.2) is the default view of the sequence viewer when a sequence list containing very large sequences is selected. It is also launched when multiple large sequences are selected in the document table.

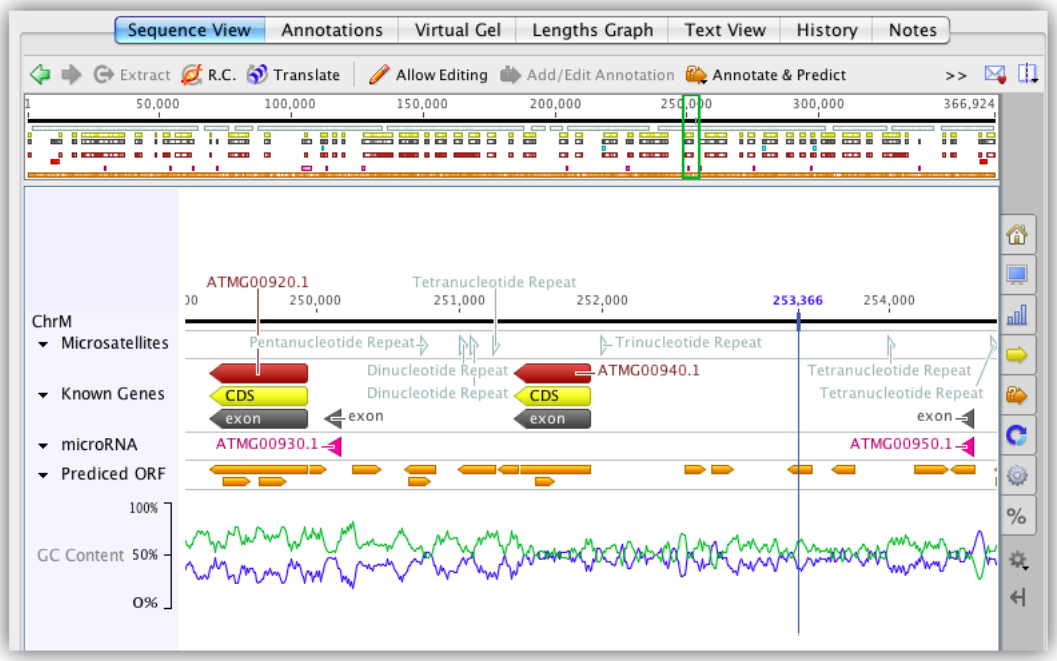


Figure 3.2: The minimap and sequence view, of a chromosome with gene and variation annotations, under the genome viewer configuration

The genome viewer provides the genome viewer selection controls, allowing for the efficient navigation of large sequences. These controls grant the ability to select individual sequences

from the sequence list as well as an extended set of zoom controls. The Go to Postion button allows for the instant navigation to a particular nucleotide coordinate for any sequence in the current document selection, using UCSC genome browser notation.

Additionally, the genome viewing configuration will display a minimap representing the currently selected sequence and it's underlying annotations. The minimap will always show a representation of the entire sequence visible in the sequence viewer. The portion of the sequence currently visible in the viewing window highlighted on the minimap, showing the relative position of the visible section to the overall sequence.

The minimap can also be used to quickly navigate around the visible sequence. Clicking on a section of the minimap will jump the sequence viewer to center on that position. Double-clicking the minimap will zoom further in on the clicked section. Finally, highlighting a section of the minimap using a click-drag-release action will display the highlighted region in the sequence viewer.

3.2.4 Colors

The colors option controls the coloring of the sequence nucleotides or amino acids. Coloring schemes differ depending on the type of sequence. For example, the "Polarity" and "Hydrophobicity" coloring schemes are available only for Protein sequences.

Similarity Color Scheme

The similarity scheme is used for quickly identifying regions of high similarity in an alignment.

In order for a column to be rendered black (100% similar) all pairs of sites in the column must have a score (according to the specified score matrix) equal to or exceeding the specified threshold.

So for example, if you have a column consisting of only K (Lysine) and R (Arginine) and are using the Blosum62 score matrix with a threshold of 1, then this column will be colored entirely black because the Blosum62 score matrix has a value of 2 for K vs R.

If you raised the threshold to 3, then this column would no longer be considered 100% similar. If the column consisted of 9 K's and 1 R, then continuing with the threshold value of 3, the 9 K's which make up 90% of the column would now be colored the dark-grey (80%→100%) range while the single R would remain uncolored.

If instead the column consisted of 7 K's and 3 R's (still with threshold 3) then 70% of the column is now similar so those 7 K's would be colored the lighter grey (60%→80%) range.

Alternatively, going back to the default threshold value of 1, and with a column consiting of 7 K's, 2 R's and 1 Y, now since the 7 K's and 2 R's have similarity exceeding the threshold whereas

the Y is not that similar to K and R, the K's and R's will be colored dark grey since they make up 90% of the column.

Hydrophobicity color scheme

This colors amino acids from red through to blue according to their hydrophobicity value, where red is the most hydrophobic and blue is the most hydrophilic. The values the color scale is based on are given in Figure 3.3. These values are taken from http://biochem.ncsu.edu/faculty/mattos/CrystallographyTutorial/AminoAcids.htm

Amino acid		Hydrophobicity	
Phe	F	1.000	**Red**
Leu	L	0.943	
Ile	I	0.943	
Tyr	Y	0.880	
Trp	W	0.878	
Val	V	0.825	
Met	M	0.738	
Pro	P	0.711	
Cys	C	0.680	
Ala	A	0.616	
Gly	G	0.501	**Purple**
Thr	T	0.450	
Ser	S	0.359	
Lys	K	0.283	
Gln	Q	0.251	
Asn	N	0.236	
His	H	0.165	
Glu	E	0.043	
Asp	D	0.028	
Arg	R	0.000	**Blue**

Figure 3.3: Hydrophobicity values for amino acids and corresponding color scale

Polarity color scheme

This colors amino acids according to their polarity as follows:

Yellow: Non-polar (G, A, V, L, I, F, W, M, P)
Green: Polar, uncharged (S, T, C, Y, N, Q)
Red: Polar, acidic (D, E)
Blue: Polar, basic (K, R, H)

3.2.5 General Options

Contains the color options (see above), check-boxes to turn on and off main aspects of the sequence view and options for what to display as the name of each sequence.

3.2.6 Display Options

Consensus

These options are available when viewing alignments. When checked, the viewer displays the `consensus` sequence with the aligned sequences. The consensus sequence has the same length (including only untrimmed bases), and shows which residues are conserved (are always the same), and which residues are variable. A consensus is constructed from the most frequent residues at each site (alignment column), so that the total fraction of rows represented by the selected residues in that column reaches at least a specified threshold. IUPAC ambiguity codes (such as R for an A or G nucleotide) are counted as fractional support for each nucleotide in the ambiguity set (A and G, in this case), thus two rows with R are counted the same as one row with A and one row with G. When more than one nucleotide is necessary to reach the desired threshold, this is represented by the best-fit ambiguity symbol in the consensus; for protein sequences, this will always be an X. In the case of ties, either all or none of the involved residues will be selected. Hence, an alignment column with only A's and G's in equal number will be represented as an R in the consensus sequence regardless of the consensus threshold.

When *ignore gaps* is checked, the consensus is calculated as if each alignment column consisted only of the non-gap characters; otherwise, the gap character is treated like a normal residue, but mixing a gap with any other residue in the consensus always produces the total ambiguity symbol (N and X for nucleotides and amino acids, respectively).

When the aligned sequences contain quality information in the form of chromatograms, you can select *Highest Quality* to calculate a majority consensus that takes the relative residue quality into account. This sums the total quality for each potential base call, and if the total for a base exceeds 60% of the total quality for all bases, then that base is called.

You can also choose to map the quality of the sequences onto the consensus. Choose *Highest* to map the quality of the highest quality base at each column onto the consensus. Select *Total* to map the sum of the contributing bases, minus the sum of the non-contributing bases. For example: if there are two G's and three A's in a column, with the G's having qualities of 16 and 24, and the As having qualities of 40, 42, and 50 respectively, then the quality of the consensus will be $(40 + 42 + 50) - (16 + 24) = 92$.

For alignments or contigs with a reference sequence, the *If no coverage call* setting can be used to control what character the consensus sequence should use when the reference sequence has no coverage. Options available are -, X/N, ? or Ref-Seq. A '?' represents an unknown character, potentially a gap. If Ref-Seq is selected, then the consensus is assigned whatever character the reference sequence has at that position. Note that if any sequence in the alignment/contig has an internal gap in it, that is still considered valid coverage at that position, and this setting will not apply.

Choose *Call N if quality below* to change consensus bases to N's if the quality is below the threshold that you set. This is particularly useful for exporting sequences to file formats which do not preserve quality (for example FASTA).

Highlighting

When *Highlight disagreements* is checked, the residues in the alignment that are identical to the consensus state for that column are grayed out. This allows you to quickly locate variable sites in the alignment.

Similarly *Highlight agreements* greys out residues that are not indentical to the consensus allowing you to quickly locate conserved sites in the alignments.

Highlight ambiguities greys out non-ambiguous residues.

Highlight gaps greys out non-gap positions.

Highlight transitions/transversions greys out residues that are not transitions/transversions compared to the consensus sequence. When highlighting transitions/transversions, it is recommended you turn on the ignore gaps consensus option or some residues may be wrongly highlighted due the consensus displaying N for sites that contain gaps and non-gaps.

Go to next disagreement/agreement/transition/transversion/ambiguity goes to the next highlighted feature as described in the previous section on highlighting.

Highlighting can be applied with reference to the consensus or a selected reference sequence.

Reverse Complement and Translation

When viewing nucleotide sequences, Geneious offers reverse complement and protein translation options.

Translations can be selected per reading frame using a range of genetic codes. They can also be created relative to selection or annotations such as CDS (Figure 3.4).

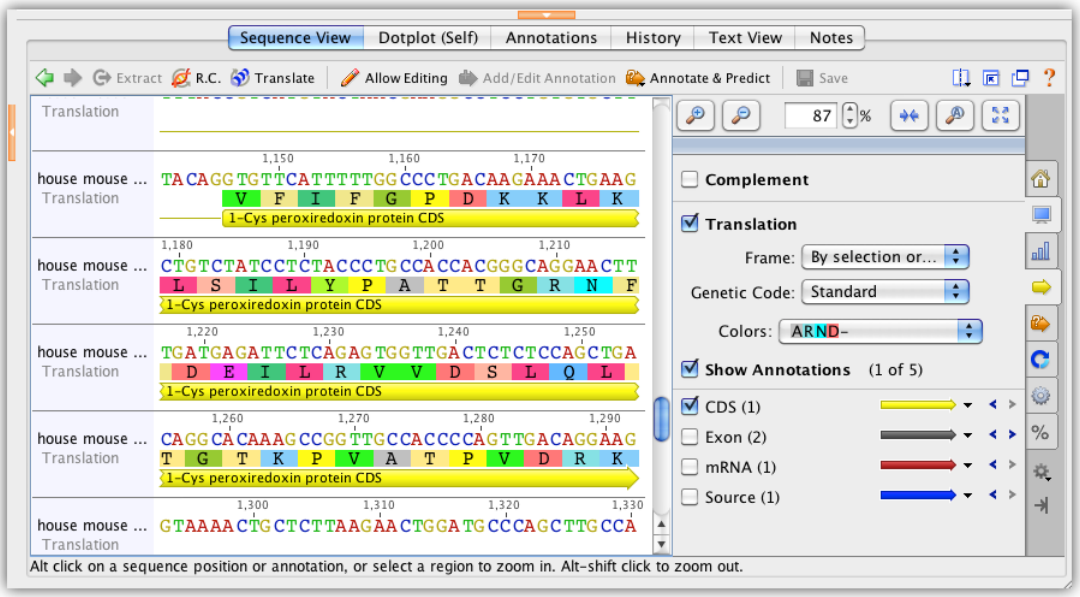


Figure 3.4: Translating a CDS

Translations can be synchronized between sequences in an alignment with reference to the individual sequences, the alignment, the consensus or a specific reference sequence.

Figure 3.5 shows an example of a DNA alignment coloured by the amino acid translation.

3.2.7 Graphs

This option is visible when viewing protein sequences, chromatogram traces, multiple sequences or sequence alignments. Turn this option on by clicking the Graph checkbox and the graph(s) will be displayed below the sequence(s). The number control to the right of each graph controls the height of that graph (in pixels). A number of graphs are available.

Protein Coding Prediction. This is available with nucleotide sequences. It runs the EMBOSS

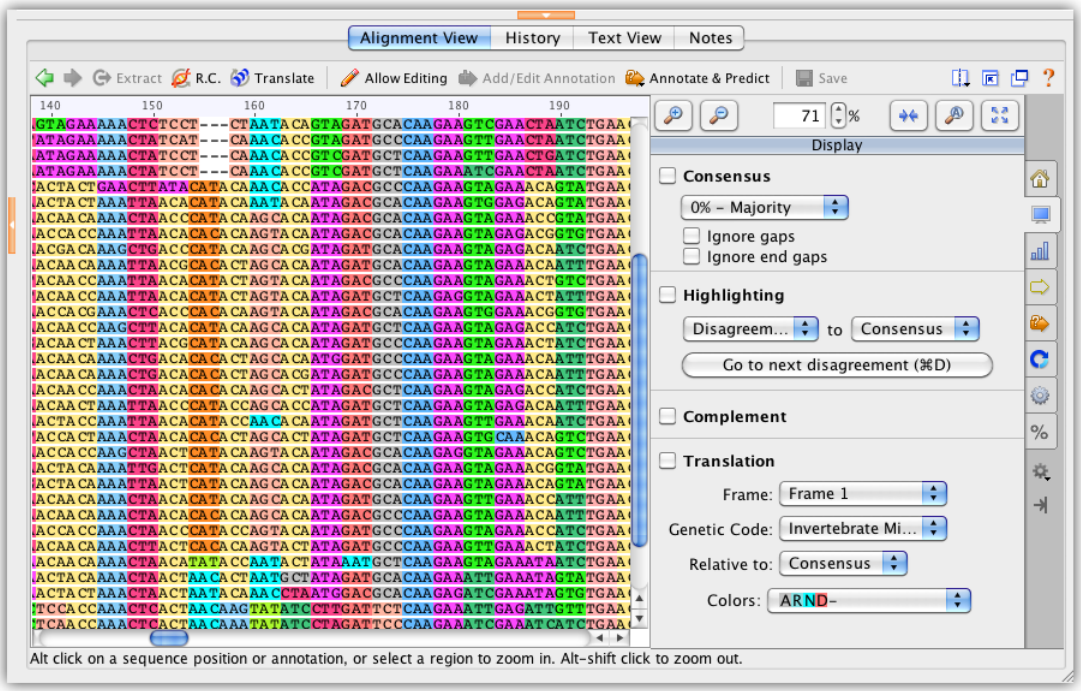


Figure 3.5: Colour an alignment by Amino Acid Translation

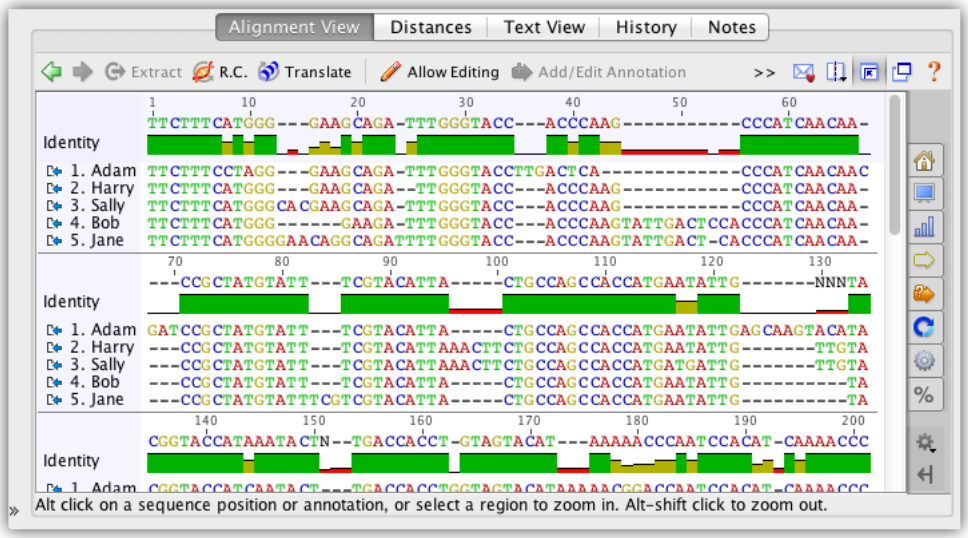


Figure 3.6: The identity graph for an alignment of nucleotide sequences

`tcode` tool and tests DNA sequences for protein coding regions using an algorithm which looks for simple and universal differences between protein-coding and noncoding DNA. The program slides a window of user-selectable size over the DNA sequence. For each window, the TESTCODE statistic is applied. The output graph indicates coding regions (green) and noncoding regions (red).

Chromatogram. This is available with chromatogram traces. It displays the four traces above the sequence, where the peak as detected by the base calling program is at the middle of the base letter. When viewing more than one chromatogram or an alignment made from chromatograms, each chromatogram can be turned on or off individually using the checkbox's below. Note that since the distance between bases as inferred from the trace varies the trace may be either contracted or expanded compared with the raw data. The vertical scale of the chromatogram can be adjusted by clicking and dragging on the graph itself. The total height of the graph can be adjusted by increasing the number displayed next to the graph on the right of the Sequence View.

Coverage. This is available on sequence alignments and contigs. The height of the graph at each position represents the number of sequence which have a non-gap character at that position. If the selected contig was created using Geneious and it contains sequences in both directions, then color coding is used to indicate whether each position is covered by reads in both directions. Green is used for regions with reads in both directions and yellow is used for regions with reads in one direction only.

The scale bar shows minimum and maximum coverage as well as a tick somewhere in between for the mean coverage.

Sequence Logo. This is available for sequence alignments. It displays a sequence logo, where the height of the logo at each site is equal to the total information at that site and the height of each symbol in the logo is proportional to its contribution to the information content. When zoomed out far enough such that he horizontal width of each site is less than one pixel, then the height is the average of the information over multiple sites. When gaps occur at at some sites, the height is scaled down further to be proportional in height to the number of non-gap residues.

Amino Acid Charge. This is available for protein sequences. It runs the EMBOSS `charge` tool to plot a graph of the charges of the amino acids within a window of specified length as the window is moved along the sequence.

Hydrophobicity. This is available with protein sequences. It displays the Hydrophobicity of the residue at every position, or the average Hydrophobicity when there are multiple sequences.

pI. pI stands for Isoelectric point and refers to the pH at which a molecule carries no net electrical charge. The pI plot displays the pI of the protein at every position along the sequence, or the average pI when multiple sequences are being viewed.

Identity. This is available for sequence alignments. It displays the identity across all sequences for every position. Green means that the residue at the position is the same across all sequences.

Yellow is for less than complete identity and red refers to very low identity for the given position (Figure 3.6).

Sliding window size. This calculates the value of the graph at each position by averaging across a number of surrounding positions. When the value is 1, no averaging is performed. When the value is 3, the value of the graph is the average of the residue value at that position and the values on either side.

Quality. This is available with enabled chromatogram traces. It displays a quality measure (typically Phred quality scores) for each base as assessed by the base calling program. The quality is shown as a shaded bar graph overlaid on top of the chromatogram. Note that those scores represent an estimate of error probability and are on a logarithmic scale - the highest bar represents a one in a million (10^{-6}) probability of calling error while the middle represents a probability of only a one in a thousand (10^{-3}).

3.2.8 Annotation Types

Some protein and nucleotide sequences come with annotations and these can be viewed within Geneious sequence viewer. Annotations can either be annotated directly on a sequence in the sequence viewer, or they can be grouped logically into tracks. A track is a collection of one or more annotation types. Tracks are stacked vertically underneath the sequence in question, with a separate line for each track and its annotations.

By clicking on the name of an annotation in the sequence view, annotations can be colored by the contents of a qualifier field. This enables the creation of annotation heatmaps by using a score value (or some other metric) stored in the qualifier of an annotation.

In the presence of annotations and tracks, the options panel includes the "Annotation Types" section (Figure 3.7). Uncheck the top check box to turn off all annotations.

Directly beneath the top check box is a filter text field. Typing a term in this field will highlight any annotations that contain the entered text in their name or qualifiers.

Annotations that are either directly annotated on the sequence or are present in multiple tracks are shown below the filter text field and have an options popup. Clicking on the preview of the annotation arrow allows you to further customise the way each type is displayed, group annotation types under new tracks as well as delete all annotations of a particular type.

Additionally, on the right of the popup button are two small left/right buttons which will move the selection in the sequence view to the next or previous instance of each annotation type. This is useful for navigating large genomes or assemblies.

Underneath this general annotation type list is the annotation type listing for the tracks present for the current sequence. Tracks with only one annotation type will show a single listing, whilst

tracks with multiple annotations will show a listing of contained annotations, segregated by the annotation type. Additionally, the Options dropdown for the individual tracks allows for sorting and coloration of tracks by contained qualifiers.

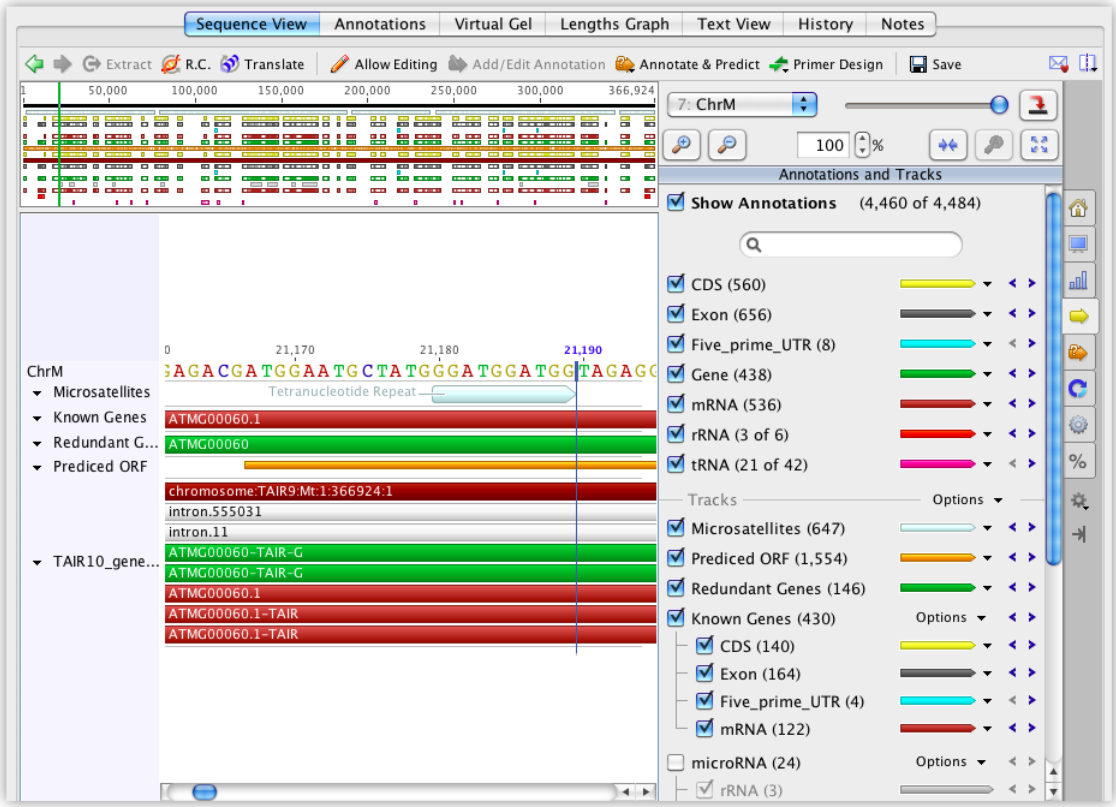


Figure 3.7: The annotations options in the sequence viewer

3.2.9 Live Annotate & Predict

This section contains any real-time annotation generators such as Find ORFs and Predict Protein Secondary Structure. Others may be available if plugins are installed.

To use one of these, turn on the check-box at the top of the generator you want to use and annotations will immediately be added to the sequence. You can then change settings for the generator and the annotations will change on the sequence in real-time as you do. You don't need to click the 'Apply' button unless you want to save the annotations to the sequence permanently.

3.2.10 Restriction Analysis

This behaves similarly to the "Live Annotate & Predict" section above. Please refer to the 10 chapter for full details.

3.2.11 Advanced

Has various options controlling the look of the sequence:

- *Wrap sequence*. This wraps the sequences in the viewing area.
- *Linear view on circular sequences*. This forces circular sequences to be shown linearly.
- *Spaces every 10 residues*. If you are zoomed in far enough to be able to see individual residues, then an extra white space can be seen every 10 residues when this option is selected.
- *Hide bases and residues*. Hides the residues/bases of the sequence and just leaves the annotations visible.
- *Show Name*. Show or hide sequence and graph names inside the sequence viewer panel.
- *Show residue numbers*. This toggles the display of the residue position number above the sequence residues.
- *Show original base numbers*. This toggles the display of the residue position numbers for the original sequence on a per sequence basis. It is only available for alignment documents and sequences that were extracted from other sequences.

- *Outline residues when zoomed out*. This adds a fine line around the sequence which can help with clarity and printing.

You can also adjust the appearance of annotations:

- *Labels*. This option changes how labels are displayed: "Inside", "Outside", "Inside or Outside" and "None".
- *Overlay on bases when zoomed out*. When only a single annotation covers a region, it will be placed on top of the sequence.
- *Compress annotations*. This option reduces the vertical height of the annotations on display. This reduces the space occupied by annotations by allowing them to overlap and increases the amount of the sequence displayed on the screen.
- *Hide excessive labels*. This will reduce screen clutter by removing annotation labels which are too frequent.

Finally, you can control the size of fonts for bases, labels, names and numbering.

3.2.12 Statistics

This displays some statistics about the sequence(s) being viewed. They correspond to the sequence/alignment/assembly being viewed or the highlighted part of the sequence/alignment/assembly. The length of the sequence or part of the sequence is displayed next to the Statistics option.

Residue frequencies. This section lists the residues for both DNA and amino acid sequences, and also for alignments and assemblies. It gives the frequency of each nucleotide or amino acid over the entire length of the sequence, including gaps. If there are gaps, then a second percentage frequency is calculated ignoring gap characters. The G+C content for nucleotide sequences is shown as well for easy reference.

The following statistics are available when viewing protein sequences:

Molecular Weight. Calculates the molecular weight of the protein using the following values for the amino acids: A=71.0788 R=156.1875 N=114.1038 D=115.0886 C=103.1388 E=129.1155 Q=128.1307 G=57.0519 H=137.1411 I=113.1594 L=113.1594 K=128.1741 M=131.1926 F=147.1766 P=97.1167 S=87.0782 T=101.1051 W=186.2132 Y=163.1760 V=99.1326 U=150.0388 O=237.3018

Isoelectric Point. Calculates the isoelectric point of the protein as per this method, but using the following values for the amino acids: D=-3.9 E=-4.1 C=-8.5 Y=-10.1 H=6.5 K=10.8 R=12.5

Extinction Coefficient. Calculates the extinction coefficient of the protein as per this paper, using the following values for the amino acids and assuming all cysteines are paired in a disulfide bridge (making cystine): C=62.5 (only counting up to an even number) W=5500 Y=1490

The following statistics are available when viewing nucleotide sequences:

Amino Acids and Codons. Calculates the distribution of Amino Acids found by translating according to the current translation options. For example if "By Selection or Annotation" is selected, then all CDS annotations will be translated and statistics presented. For codon usage statistics, the frequency of all 64 codons (with their associated amino acid) will be displayed. If any CDS contains non-standard start codons then some of the 64 codons may be split into 2 entries based on whether they translate to methionine or their standard translation.

The following statistics are available when viewing multiple sequences:

Identical sites. When viewing alignments or assemblies this considers only those columns in the alignment that have at least 2 nucleotides/amino acids/gaps that are not free end gaps and are not columns consisting entirely of gaps. A column not meeting this requirement is not even counted as non-identical for the percentage calculation. A column meeting this requirement is considered identical if it contains no internal gaps and all the nucleotides/amino acids are identical. Ambiguitity characters are not interpreted, so a nucleotide columm of A and R is not considered idential.

Pairwise % Identity. When viewing alignments or assemblies this gives the average percent identity over the alignment. This is computed by looking at all pairs of bases at the same column and scoring a hit (one) when they are identical, divided by the total number of pairs. Ambiguity charactres are interpreted, meaning a nucleotide A vs a nucleotide R is considered to have 50% identity.

Confidence (mean). When viewing chromatograms this gives the mean of the confidence scores for the currently selected base calls. Confidence scores are provided by the base calling program (not Geneious) and give a measure of quality (higher means a base call is more likely to be correct). An untrimmed value is also displayed if the selected region contains trims.

Expected Errors. When viewing chromatograms, this gives the approximate number of errors that are statistically expected in the currently selected region. This is calculated by converting the confidence score for each base call in to the error probability and summing across the region. This also has a value for the untrimmed selection if the region contains trims.

[Ungapped] Lengths of Sequences. Displays the mean, standard deviation, minimum and maximum of the lengths of the sequences.

Coverage of Bases. When viewing a contig assembly this gives the mean, standard deviation, minimum and maximum of the coverage of each base in the consensus sequence. If your contig has a reference sequence, then the percentage of the ungapped reference sequence that is covered by at least 1 read is also displayed.

Rough Tm. A rough calculation of the melting point for a nucleotide sequence using the following calculations:

If the sequence is less than 14bp in length, $RoughTm = 4 \times GCcount + 2 \times ATcount$

If the sequence is greater than 13bp in length, $RoughTm = 64.9 + 41 \times (GCcount - 16.4) \div length$

3.2.13 The sequence viewer toolbar

The top of the sequence viewer panel shows a toolbar containing several actions. Some of them operate on a part of a sequence or alignment. There are several ways to make such a selection.

- *Mouse dragging*. Click and hold down the left mouse button at the start position, and drag to the end position. By using the Ctrl (Windows/Linux) or ⌘ (Mac) keys it is possible to select multiple regions of a sequence or alignment.
- *Select from annotations* When annotations are available, click on any annotation to select the annotated residues. As with mouse dragging, multiple selections are supported.
- *Click on sequence name*. This will select the whole sequence.
- *Select all*. Use the keyboard shortcut Ctrl+A (⌘+A on Mac) to select everything in the panel.

The available actions are,

Extract Extract the selected part of a sequence or alignment into a new document.

Reverse Complement Reverse sequence direction and replace each base by its complement. This is available only for nucleotide sequences.

Translate. Translate DNA into protein. Clicking on this choice brings up a list of genetic codes that can be used. Choose the appropriate one and click OK. This is available only for nucleotide sequences.

Allow Editing, Add/Edit Annotation, Annotate & Predict and Save

3.2.14 Editing sequences and alignments

To edit sequence(s) or an alignment click the "Allow Editing" toolbar button. After selecting a residue or a region you can either type in the new contents or use any of the standard editing operation such as Copy (Ctrl/⌘+C), Cut (Ctrl/⌘+X), Paste (Ctrl/⌘+V), Paste Without Annotations (Shift+Ctrl/⌘+V), Paste Reverse Complement and Undo (Ctrl/⌘+Z). All operations are under the main "Edit" menu.

Selecting a region enables the "Add/Edit Annotation" button as well, which opens an annotation entry dialog. Enter an annotation name and select a existing type or type a new one. Click on "More Options" to enter additional properties for that annotation. Double click on an existing annotation to edit it or right-click (Ctrl+click on Mac OS X) to display a pop-up menu to delete annotations. You can also copy an annotation from one sequence to another from the pop-up menu.

When editing an alignment it is possible to select a region (which may span several sequences) and drag it to the left or right. Dragging will either move residues over existing gaps or open new gaps when necessary. Dragging a selection consisting entirely of gaps moves the gaps to the new location.

To quickly select a single residue, double-click on it. Triple clicking will select a block of residues within a single sequence. Quadruple clicking selects a block of residues in multiple sequences.

The Shift and Ctrl (Alt/Option on a Mac) keys can be combined with the keyboard arrow keys to select sequence and alignment regions. The Shift key extends the current selection and holding down the Ctrl (Alt/Option on a Mac) key while pressing the keyboard arrow is equivalent to pressing it 10 times. These can be used together. For example, in an alignment if you have a region of one sequence selected, and would like to select the same region in all sequences, then you could press Ctrl-up until you reach the first sequence, and then press Ctrl-Shift-down and few times until all sequences are selected.

Sequences can be reordered within an alignment by clicking the sequence name and dragging.

Sequences can be removed from an alignment by right-clicking (Ctrl+click on Mac OS X) on the sequence name and choosing the "remove sequence" option. Alternatively, select the entire sequence (by clicking on the sequence name) and press the delete key.

To delete a region of an alignment, select the region and press the delete or backspace key. Normally this will move residues on the right into the deleted area. By holding down the Shift key while deleting, residues on the left will be moved into the deleted area instead. Similarly, holding down the Shift key when inserting will push residues to the left instead of right.

Shift+click on two restriction site annotations in the sequence view to select the region between their cut sites on the forward strand.

After editing is complete, click 'Save' to permanently save the new contents.

3.2.15 The Pop up menu in the sequence viewer

The toolbar actions are available via a pop-up menu as well. Right-click (Ctrl+click on Mac OS X) on any sequence, partly highlighted sequence, or annotation to show the various options. The pop-up menu contains the "Copy residues" action (keyboard Ctrl+C) to copy the selected residues to the system clipboard.

3.2.16 Printing a sequence view

To print a sequence view, go to "File" → "Print" and click "OK". The view is printed without the options panel. It is recommended to turn on "Wrap sequence" and deselect "Colors" before printing. Wrapping prints the sequence as seen in the sequence viewer and the font size is chosen to fill the horizontal width of the page.

3.3 Annotation Viewer

The "Annotations" tab appears whenever sequences containing annotations are selected. It displays each annotation as a row in a table, with columns corresponding to the qualifiers for the annotations. Selection of annotations is synchronised with other viewers, such as the sequence viewer and dotplot.

3.3.1 Menu

- *Types* allows selecting a subset of types for display in the table.

  The "Select One" button in the menu is a quick way to view just one type while also selecting the relevant columns for that type. Relevant columns are deemed to be ones where at least one annotation of that type has a value for the column.

- *Columns* allows control over which columns are visible in the table.

- *Export table* exports the visible rows and columns to a CSV (comma-separated values) file.

- *Extract* extracts the region of the selected annotation into a new document.

- *Translate* translates the nucleotides in the region of the selected annotation into amino acids, allowing selection of the appropriate translation table and frame.

- *Filter* text in this field is used to filter the table. Filtering is only done against the currently visible columns for each annotation.

3.4 Dotplot viewer

This is a special viewer that appears when one or two sequences are chosen. A dotplot compares two sequences to find regions of similarity. Each axis (X and Y) on the plot represents one of the sequences being compared (Figure 3.8). For more information on dotplots, see section 4.3.

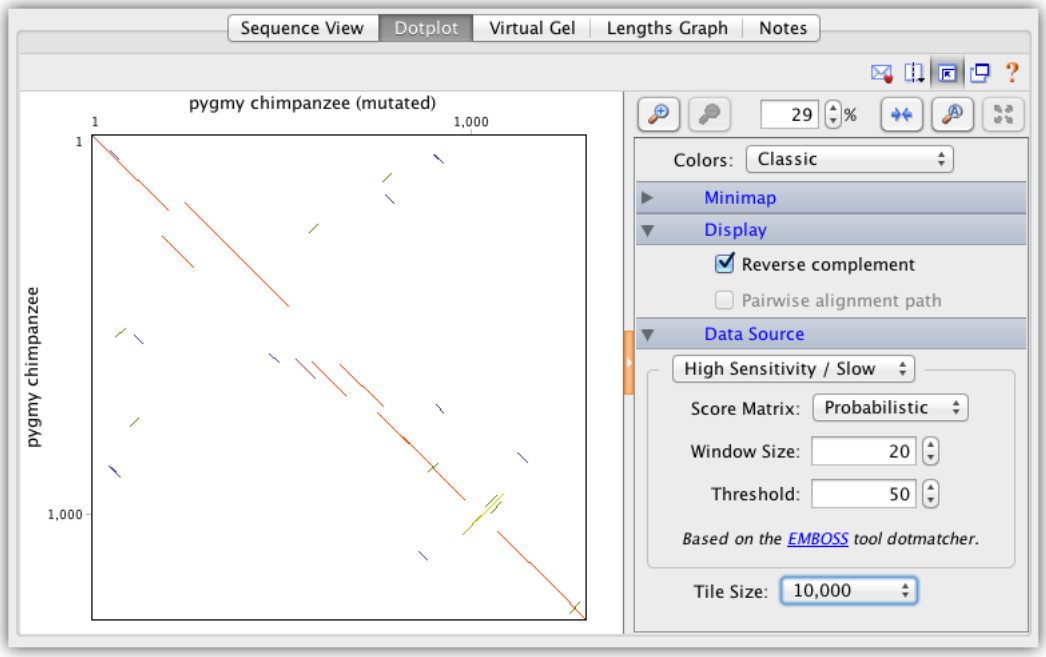


Figure 3.8: A view of dotplot of two sequences in Geneious

3.5 RNA/DNA secondary structure fold viewer

This viewer will appear when the selected nucleotide sequence is less than 3000bp long. If the sequence is DNA, the tab will be labelled 'DNA Fold' and if it is RNA it will be labelled 'RNA Fold' (Figure 3.9)

The fold prediction is performed by the Vienna package `RNAfold` tool. Information on the options for this tool can be found at the following web page: `http://www.tbi.univie.ac.at/~ivo/RNA/RNAfold.html`.

The "View Options" allow you to turn off/on and color the bases, flip the coordinates, highlight the start (blue) and end (red) of the sequence and rotate the model. As with other viewers, you can zoom in on the model and drag the view around or use the scrollwheel using the same keyboard modifiers as the sequence viewer. Selection is synchronized between the sequence view and the fold view. In addition, when in split view mode, the fold viewer will scroll to the selected area when zoomed in.

By default, color by probability is used where red bases are the ones with the strongest probability of the bases being paired with each other in paired regions, or being unpaired in unpaired regions. Green is the middle ground and blue is the lowest probability. Color by probability is only available when using the Partition Function.

The "Compute Options" will rerun `RNAfold` when you change their settings so depending on the size of the sequence there may be a noticeable recompute time.

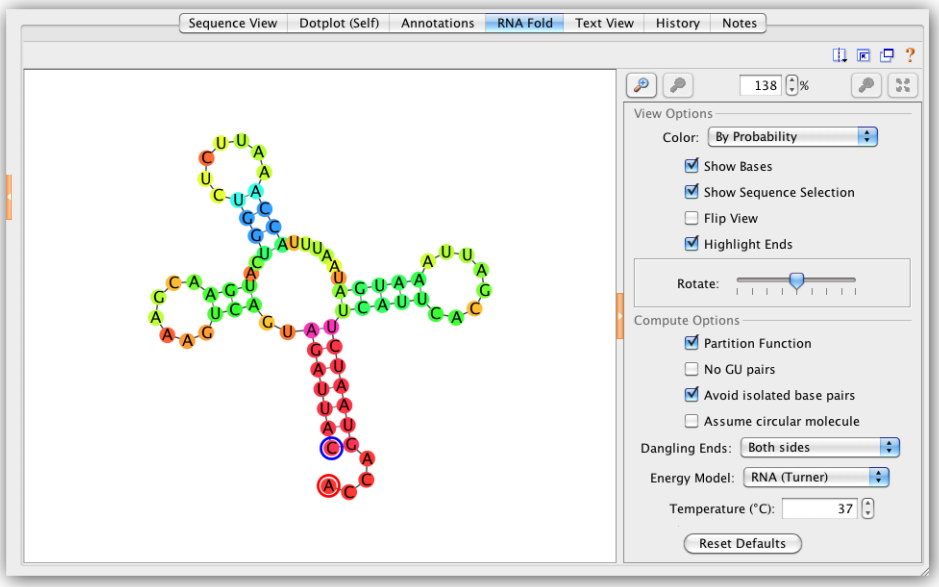


Figure 3.9: A view of an mRNA secondary structure prediction in Geneious

3.6 3D structure viewer

For molecular structure documents, such as PDB documents, this displays an interactive three dimensional view of the structure.

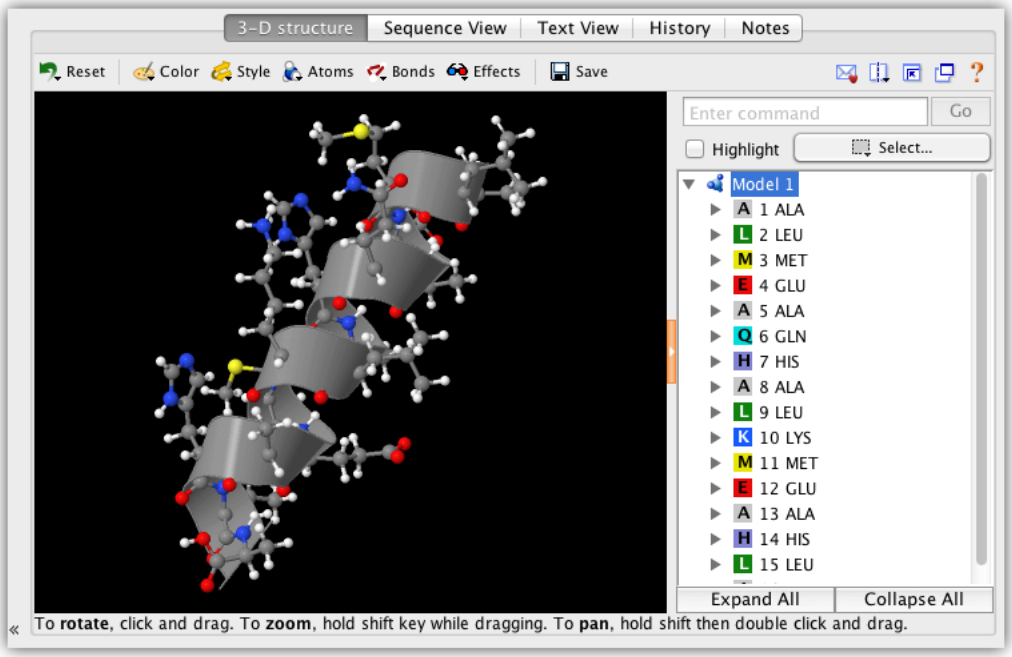


Figure 3.10: A view of a 3D protein structure in Geneious

3.6.1 Structure View Manipulation

- Click and drag the mouse to rotate the structure.
- Hold the Alt or Shift key then click and drag to zoom in/out
- Hold the Ctrl key then right-click and drag to pan, or, if you are using a Mac, click and hold, press Ctrl and Alt/Option then drag to pan.

3.6.2 Selection Controls

To the right of the structure are controls that let you control the selected part of the structure.

- If the structure you are viewing contains more than one model, the *model* combo box will you choose between them.
- The *select* button lets you select all, none or the nonselected region of the structure, as well as by element, group type or secondary structure.
- The *highlight selected* checkbox lets you select whether to highlight the selected atoms in the structure view.
- The *structure tree* shows the atoms in the structure in a tree format. Click on regions in the tree to select thoses regions. You can also Shift-click and Ctrl-click to select mutliple regions at once.
- The *command box* lets you type in arbitrary jmol scripting commands. To see some examples, select one of the pre-populated options in the box's drop-down. For a complete description of the commands you can use, see http://www.stolaf.edu/academics/chemapps/jmol/docs.

3.6.3 Display Menu

At the top of the viewer is the display menu. Here you can modify the appearance of the structure.

- *Reset* lets you reset the position of the structure, reset the appearance of the structure to the default, or reset the appearance of the structure to its appearance when it was last saved.
- *Color* lets you change the color scheme of the selected region of the atom.
- *Style* lets you change the style of the selected region of the molecule eg to spacefill or cartoon view.
- *Atoms* lets you hide atoms or change their size in the selected region of the molecule. You can also choose whether to show hydrogen atoms and atom symbols.
- *Bonds* lets you hide bonds or change their size in the selected region of the molecule. Covalent/ionic bonds, hydrogen bonds and disulfide bonds can be affected separately.
- *Effects* lets you toggle spin, antialiasing, stereo and slabbing effects for the whole molecule.
- *Save* saves the current appearance of the molecule.

3.7 Tree viewer

The tree viewer provides a graphical view of a phylogenetic tree (Figure 3.11). When viewing a tree a number of other view tabs may be available depending on the information at hand. The "Sequence View" tab will be visible if the tree was built from a sequence alignment using Geneious. The "Text View" shows the tree in text format (Newick).

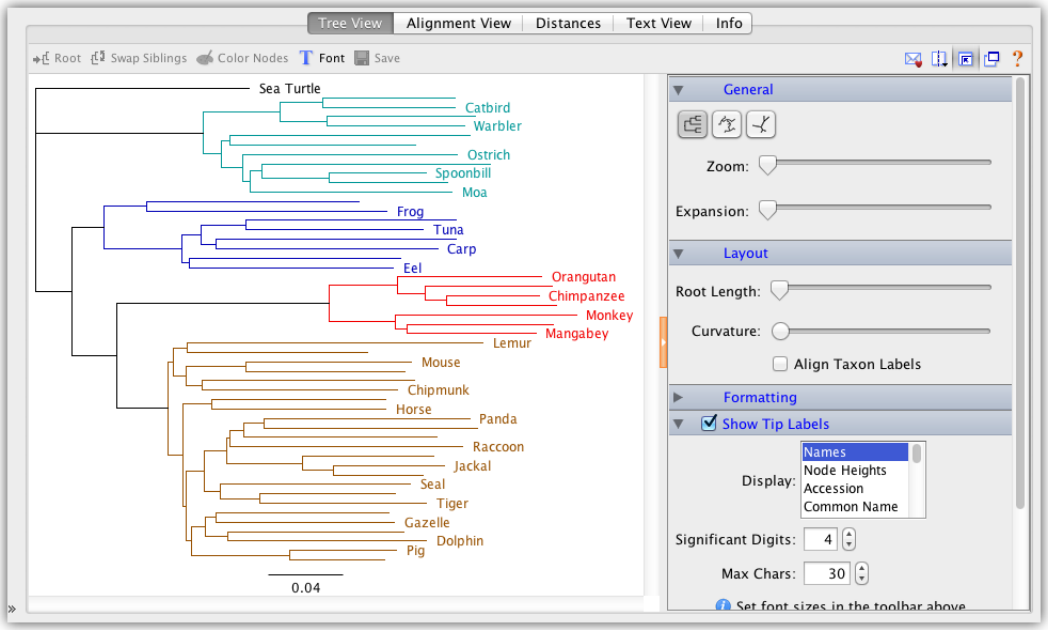


Figure 3.11: A view of a phylogenetic tree in Geneious

There are a number of options for the tree viewer.

3.7.1 Current Tree

If you are viewing a tree set, this option will be displayed. Select the tree you want to view from the list.

3.7.2 General

"General" has 3 buttons showing the different possible tree views: rooted, circular, and unrooted. The "Zoom" slider controls the zoom level of the tree while the "Expansion" slider expands the tree vertically (in the rooted layout).

3.7.3 Info

For a consensus tree, the info box displays the consensus method used to build the tree. For a topology, it also shows what percentage of the original trees have the topology of the displayed tree.

3.7.4 Layout

This has different options depending on the layout that you select above:

- *Root Length* Sets the length of the visible root of the tree *(Rooted and Circular views)*
- *Curvature* Adds curvature to the tree branches *(Rooted view only)*
- *Align Taxon Labels* Aligns the tip labels to make viewing a large tree easier *(Rooted view only)*
- *Root Angle* Rotates the tree in the viewer *(Circular and Unrooted views)*
- *Angle Range* Compresses the branches into an arc *(Circular view only)*

3.7.5 Formatting

There are a range of formatting options.

Transform branches allows the branches to be equal like a cladogram, or proportional. Leaving it unselected leaves the tree in its original form.

Ordering orders branches in increasing or decreasing order of length, but within each clade or cluster.

Show root branch displays the position of the root of the tree *(has no effect in the unrooted layout)*.

Line weight can be increased or decreased to change the thickness of the lines representing the branches.

Auto subtree contract automatically contracts subtrees when there is not enough space on-screen to display them nicely.

Show selected subtree only shows only the part of the tree that is selected (or the entire tree if there is no selection).

If you are unfamiliar with tree structures, please refer to Figure 3.12 for the following options.

Show tip labels. This refers to labels on the tips of the branches of the tree.

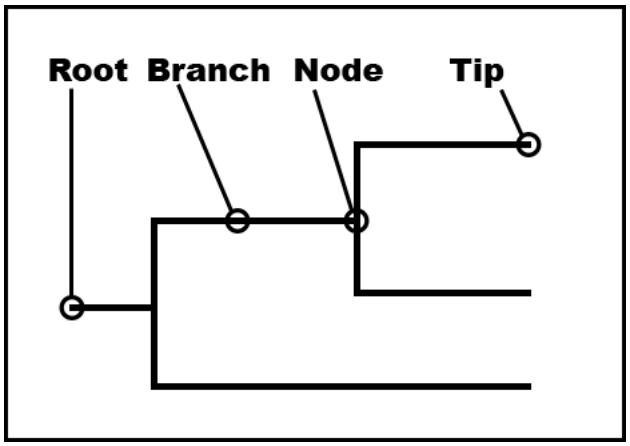


Figure 3.12: Phylogenetic tree terms

Show node labels. This refers to labels on the internal nodes of the tree.

Show branch labels. This refers to the branches of the tree.

Each of the three above options has fields that you can set to customise what the labels display.

- "Display" allows you to select what information the labels display. Branch Labels have fixed settings, but you can select what the Tip Labels display (either Taxon Names, Node Heights, Sequence Names, or a number of other options depending on the tree you are viewing). If you are viewing a consensus tree, you can also display consensus support as a percentage on node labels.
- You can use "Font" to change the size of the labels. The tree viewer will shrink the font size of some labels if they cannot all fit in the available space. "Minimum Size" specifies the minimum size that the tree viewer is allowed to shrink the label font to.
- "Significant Digits" sets how many digits to display if the value the node is displaying is numeric.

Show scale bar. This displays a scale bar at the bottom of the tree view to indicate the length of the branches of the tree. It has three options: "Scale range", "font size" and "line weight". Setting the scale range to 0.0 allows the scale bar to choose its own length, otherwise it will be the length that you specify.

3.7.6 Node Interaction

You may click on a node in the tree viewer to select the node and its clade. Double-click the node to collapse/un-collapse the clade in the view. Once you have selected a clade in the view,

you may edit the tree *(see below)*

3.7.7 The Toolbar

The buttons on the toolbar along the top of the viewer allow you to edit the tree.

If you are viewing a tree made from an alignment, the "View Sequences" button allows you view the selected nodes in the sequence viewer.

The "Root" button allows you to re-root the tree on the selected node.

The "Swap Siblings" button allows you to swap the position of the sibling clades of the selected node.

3.8 Info Viewer

The info viewer contains information about the document, including notes, editing history and linked documents. This information is organized into three tabs: properties, history and lineage.

The notes tab allows you to add notes to your document as meta-data (see section 2.8).

The history viewer displays the complete history for a selected document. The exact information displayed is flexible, but is made up of entries each of which will always include the time and user responsible for the edit. An entry may also reference other documents via hyperlinks, and has the ability to display a recreation of the options used. Saving of history can be disabled for performance or privacy reasons by going to the Appearance and Behaviour tab in Preferences, see section 2.9.

The lineage view tab contains a list of the linked (actively or otherwise) documents in Geneious (see section 3.9.2).

3.9 Parents and Descendants

Many documents in Geneious are the output of an operation run on a set of input documents. The input documents of the operation are known as the **parents** of the output, and the output documents the **descendants** (or **children**) of the input. Those parent documents may themselves be the descendants of other documents, each with their own parents, and so on. In many situations it is useful to preserve this hierarchy, so that future alterations, for example the re-calling of a base, or the addition of a new annotation, can be transferred downstream to the molecules affected by this change in a parent.

An **active link** between a child and its parents means that when you modify any of the parent documents, you will be given the choice of propagating these changes to the child. When this modification affects a part of the parent involved in creating the child, the change will be immediately visible in the child. Modifications include things like editing the residues of a sequence, adding new annotations, or changing the meta-data associated with the document.

Propagating a change to a parent document causes Geneious to rerun every operation that links that parent actively to one or more child documents, with the altered parent document (and any other parents) as input. Geneious stores the options that the operation was originally run with so that it can reproduce the original operation's conditions exactly, and afterwards matches up the newly regenerated child documents with any former children, and replace their contents where possible.

Occasionally, one or more of the parent documents has been altered to a point where an operation can no longer be rerun, or a necessary parent document is inaccessible. In this case, Geneious will inform you of the failure, and attempt to be as specific as possible about the cause of the failure (Figure 3.13)

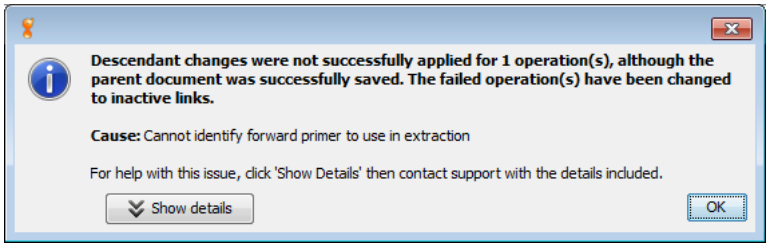

Descendant changes were not successfully applied for 1 operation(s), although the parent document was successfully saved. The failed operation(s) have been changed to inactive links.
Cause: Cannot identify forward primer to use in extraction
For help with this issue, click 'Show Details' then contact support with the details included.
Show details
OK

Figure 3.13: Failure to propagate an Extract PCR product operation due to a missing forward primer

Inactive links do not propagate changes from parent to child. Inactive links are created in two different ways; firstly, when you choose not to propagate changes, that active link becomes *temporarily* inactive. Secondly, if an operation does not support creation of active links, or was told not to create them, all links between its parents and children will be *permanently* inactive. All operations in Geneious at least create inactive links.

The following operations in Geneious can produce actively linked documents:

- Cloning: Digest into Fragments...
- Cloning: Insert into Vector...
- Cloning: Ligate sequences...
- Cloning: Gateway
- Primers: Extract PCR product

- Sequence Viewer: Extract
- Sequence Viewer: Translate

Note: Extract and Translate will not create active links by default. To do so, you must select "Actively link source and extracted documents" checkbox in the relevant dialog (see Figure 3.14), otherwise they will be created with permanently inactive links.

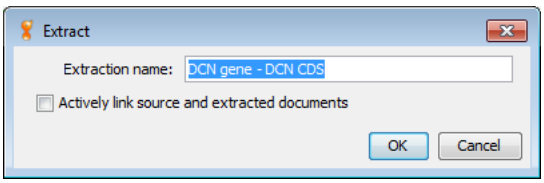


Figure 3.14: Extract dialog with active link checkbox

3.9.1 Editing Linked Documents

When you make changes to a document that is the parent of another document, you will be given the opportunity to either propagate the changes, deactivate the link (which can later be reactivated, see Lineage View, Section 3.9.2), or save the changed document as a new copy (Figure 3.15). You may also simply back out of this process by choosing to cancel, which will return you to your unsaved changes. Note that if you choose to deactivate the link, this dialog will not be displayed upon subsequent saves of the parent document, unless the link is reactivated again at some future time.

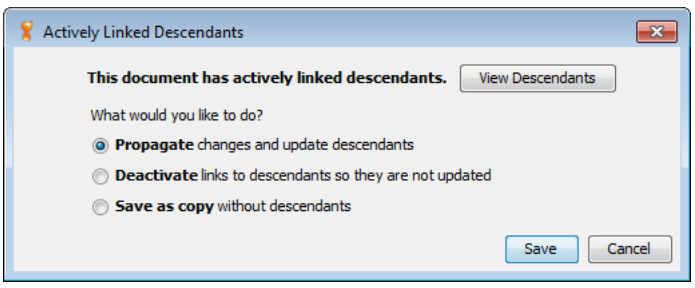

Actively Linked Descendants
This document has actively linked descendants.
View Descendants
What would you like to do?
Propagate changes and update descendants
Deactivate links to descendants so they are not updated
Save as copy without descendants
Save
Cancel

Figure 3.15: Actively Linked Descendants dialog

In order to aid with your decision making, the dialog allows you to view the document's descendants in a smaller, cut down version of the Lineage View. Pressing the "View Descendants" button will bring up this view (Figure 3.16).

When you choose to begin editing a document with actively linked **parents** in the Sequence View, you will immediately be warned that in order to save your changes you will need to

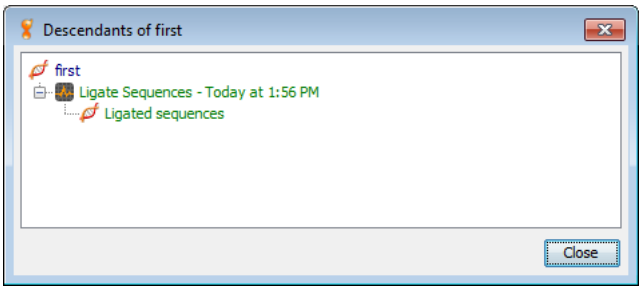


Figure 3.16: Descendants view

deactivate this link. Similarly to the Actively Linked Descendants view, you will be given the opportunity to view the document's lineage. Editing a document that is a descendant of other documents is usually unintentional; however, in some circumstances you may simply be interested in the output documents of an operation (not the parent-descendant relationship), and as such you may hide this dialog (Figure 3.17).

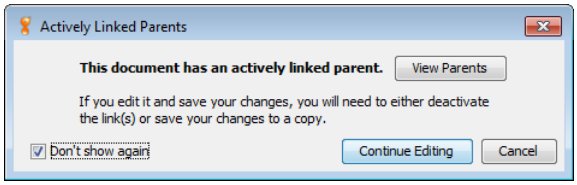

Actively Linked Parents
This document has an actively linked parent.
View Parents
If you edit it and save your changes, you will need to either deactivate the link(s) or save your changes to a copy.
Don't show again
Continue Editing
Cancel

Figure 3.17: Actively Linked Parents dialog

Upon conclusion of your editing, you will again be prompted to either deactivate links or save a copy.

3.9.2 The Lineage View

Every document that is linked (actively or otherwise) to another document has a tab called "Lineage" in the Info View tab. The lineage view allows you view parent-descendant relationships, manage links, and navigate between documents (Figure 3.18).

All active links appear as green text, whilst inactive links appear as black text and the document currently being viewed (and which is the root of the parents tree and the descendents tree) appears in blue. Each's document's name is displayed along with an icon (similar to the document table) denoting what type of sequence it is.

Also displayed in the viewer are the operations that generated each set of children, along with the time at which the operation was run and the type of operation. If preferred, these operations can be hidden by unchecking the "Show Operations" checkbox, providing a layout which is

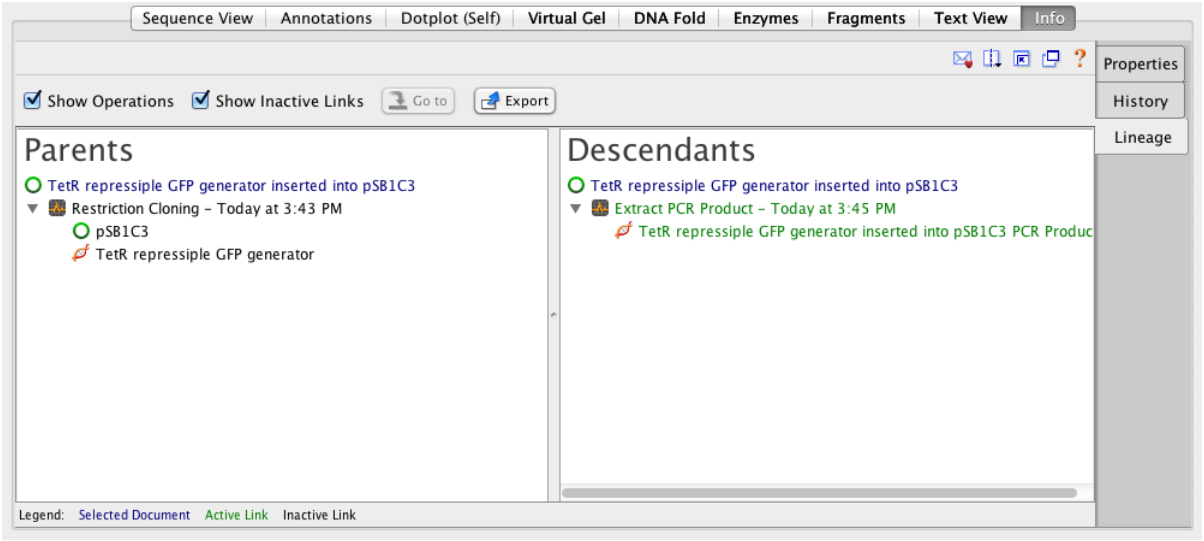


Figure 3.18: The Lineage View

akin to Vector NTI®. You can also choose to view only inactive links by unchecking the "Show Inactive Links" checkbox. **This will hide all inactively linked documents, as well as *those* documents' parents or descendants**. This means that you will only be viewing documents that are directly affected by one currently being viewed.

You can reactivate temporarily deactivated links from the view by right-clicking (Windows, Linux) or control-clicking (MacOS) on a document and choosing "Activate link to parent" from the context menu. Alternatively you can reactivate links to all children at once by choosing "Show Operations" and right- or control-clicking, then selecting "Reactivate all links for this operation". You may also manually deactivate links in this fashion (Figure 3.19).

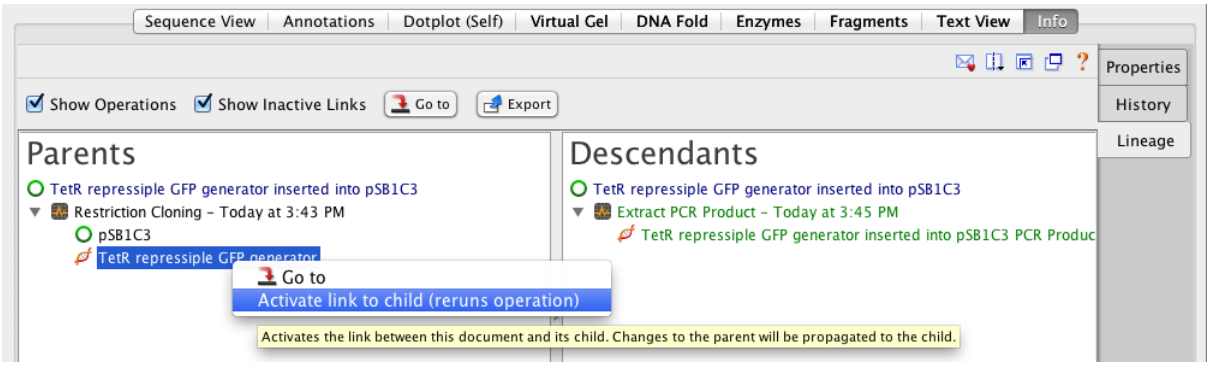


Figure 3.19: Context Menu

Note: reactivating links immediately reruns the operation; depending on the size and type of

the operation, this can be time consuming. Also note that reactivating will cause any unsaved changes to any direct or indirect descendants to be overwritten, since this involves a complete recompute from the parent documents. You will be warned about this before Geneious allows you to reactivate.

Finally, you may export the currently selected document (highlighted in blue in the view) directly, via the "export button". Doing so will bring up a dialog (Figure 3.20). From here you can choose to export parents or descendants only, or both, as well as choose to export only those documents that are actively linked in the hierarchy. Similarly to how unchecking the "Show Inactive Links" checkbox works, unchecking "Inactively linked documents" here will mean that the export will stop as soon as it finds in inactively linked parent or descendant (depending on the relevant direction), and stop exporting down that branch of the lineage.

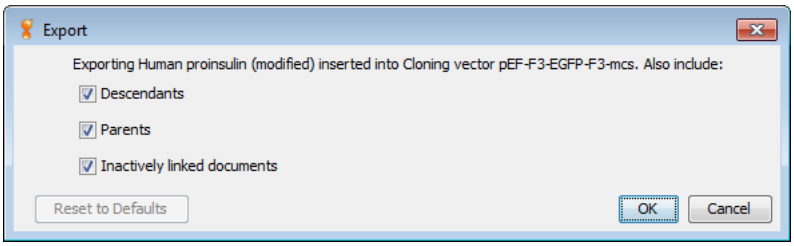


Figure 3.20: Export Dialog

3.10 The Chromatogram viewer

The Chromatogram viewer provides a graphical view of a the output of a DNA sequencing machine such as Applied Biosystems 3730 DNA analyzer. The raw output of a sequencing machines is known as a `trace`, a graph showing the concentration of each nucleotide against sequence positions. The raw trace processed by a "Base Calling" software which detects peaks in the four traces and assigns the most probable base at more or less even intervals. Base calling may also assign a quality measure for each such call, typically in terms of the expected probability of making an erroneous call.

Sequence Logo. When checked, bases letters are drawn in size proportional to call quality, where larger implies better quality or smaller chance of error. Note that the scale is logarithmic: the largest base represents a one in a million (10^{-6}) or smaller probability of calling error while half of that represents a probability of only a one in a thousand (10^{-3}).

Mark calls. Draw a vertical line showing the exact location of the call made by the base calling software.

Layout. Options controlling layout and view. Those include X and Y axis scaling, size of largest base letter (when Sequence logo is on) and minimum size of base letter (to prevent bases of low

quality becoming unreadable).

3.11 The PDF document viewer

To view a `.pdf` document either double click on the document in the Documents Table or click on the "View Document" button. This opens the document in an external PDF viewer such as Adobe Acrobat Reader or Preview (Mac OS X). On Linux, you can set an environmental variable named "PDFViewer" to the name of your external PDF viewer. The default viewers on Linux are `kpdf` and `evince`.

3.12 The Journal Article Viewer

This viewer provides two tabs: "Text View" and "BibTex". "Text view" displays the journal article details including the abstract. The text contains a link to the original article through Google Scholar below the title and authors (Figure 3.21). BibTex is the standard LaTeX bibliography reference and publication management data format. LaTeX is a common program used to create formatted documents including this one. The information in the BibTex screen can be exported for use in LaTeX documents.

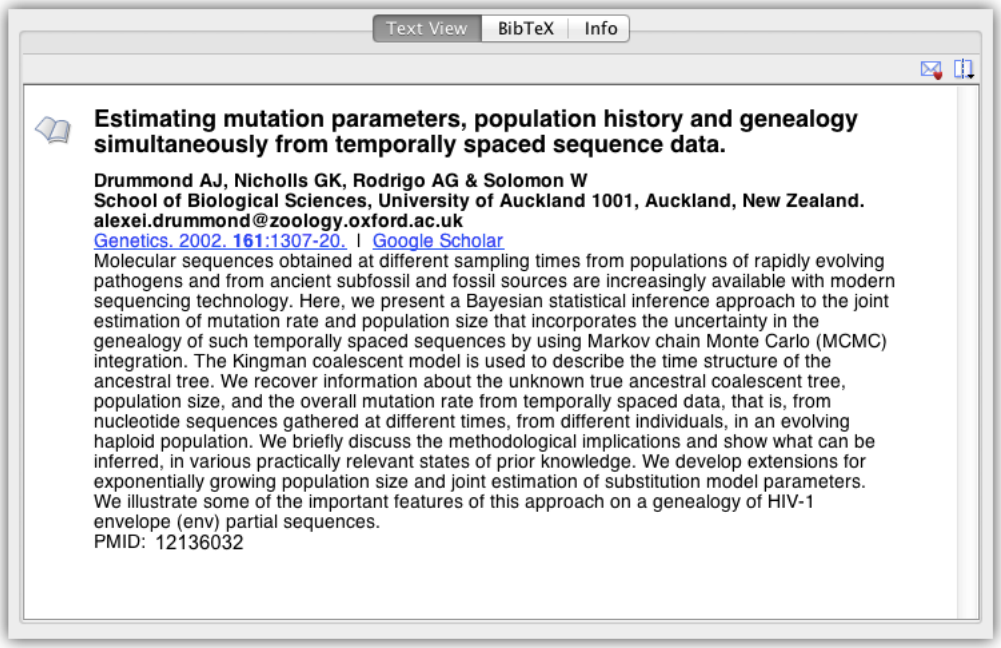

Text View
BibTeX
Info
Estimating mutation parameters, population history and genealogy simultaneously from temporally spaced sequence data.
Drummond AJ, Nicholls GK, Rodrigo AG & Solomon W
School of Biological Sciences, University of Auckland 1001, Auckland, New Zealand.
alexei.drummond@zoology.oxford.ac.uk
Genetics. 2002. 161:1307-20. | Google Scholar
Molecular sequences obtained at different sampling times from populations of rapidly evolving pathogens and from ancient subfossil and fossil sources are increasingly available with modern sequencing technology. Here, we present a Bayesian statistical inference approach to the joint estimation of mutation rate and population size that incorporates the uncertainty in the genealogy of such temporally spaced sequences by using Markov chain Monte Carlo (MCMC) integration. The Kingman coalescent model is used to describe the time structure of the ancestral tree. We recover information about the unknown true ancestral coalescent tree, population size, and the overall mutation rate from temporally spaced data, that is, from nucleotide sequences gathered at different times, from different individuals, in an evolving haploid population. We briefly discuss the methodological implications and show what can be inferred, in various practically relevant states of prior knowledge. We develop extensions for exponentially growing population size and joint estimation of substitution model parameters. We illustrate some of the important features of this approach on a genealogy of HIV-1 envelope (env) partial sequences.
PMID: 12136032

Figure 3.21: Viewing bibliographic information in Geneious

Chapter 4

Analysing Data

4.1 Literature

Geneious allows you to search for relevant literature in NCBI's PubMed database. The results of this search are summarized in columns in the Document Table and include the PubMed ID (PMID), first and last authors, URL (if available) and the name of the Journal. When a document is selected, the abstract of the article is displayed in the Document Viewer along with a link to the full text of the document if available, and a link to Google Scholar, both below the author(s) name(s).

Note: If the full text of the article is available for download in PDF format, it can also be stored in Geneious by saving it to your hard drive and then importing it. This will allow full-text searches to be performed on the article.

As well as the abstract and links, Geneious also shows the summary of the journal article in BibTex format in a separate tab of the Document Viewer. This can be imported directly into a LaTeX document when creating a bibliography. Alternatively, a set of articles in Geneious can be directly exported to an EndNote 8.0 compatible format. This is usually done when creating a bibliography for Microsoft Word documents.

4.2 Sequence data

Basic techniques, such as dotplots and pairwise alignments, can be used to study the relationships between two sequences. However, as the number of sequences increases, methods for determining the evolutionary relationships between them become more complicated.

When analyzing more than two sequences, there are some common steps to determine the ancestral relationships between them. The following sections outline the basic tools for prelim-

inary sequence analysis: dotplots, sequence alignment and phylogenetic tree building.

4.3 Dotplots

A dotplot compares two sequences against each other and helps identify similar regions [14]. Using this tool, it can be determined whether a similarity between the two sequences is global (present from start to end) or local (present in patches).

The Geneious dotplot offers two different comparison engines based on the EMBOSS `dottup` and `dotmatcher` programs. The former is much faster but less sensitive than the latter. More information on these programs can be found by going to `http://emboss.sourceforge.net`.

When viewing a pairwise alignment you can activate the path which shows where the pairwise alignment runs through the dotplot. Also, for nucleotide comparisons you can show the reverse complement.

4.3.1 Viewing Dotplots

To view a dotplot in Geneious, select two nucleotide or protein sequences in the Document Table and select Dotplot Viewer in the Document Viewer Panel (Figure 3.8). The Dotplot Viewer allows you to zoom in and out, and to customize sensitivity of the comparison.

If a single nucleotide or protein sequence is selected then the dotplot is also available. In this case it shows a comparison of the sequence to itself.

The dotplot comparison of two sequences is drawn from top-left to bottom-right in and offers a selection of different color schemes. There is also a minimap available which aids navigation of large dotplots by showing the overall comparison and a box indicating where the dotplot window sits.

4.3.2 Interpreting a Dotplot

- Each axis of the plot represents a sequence.
- A long, largely continuous, diagonal indicates that the sequences are related along their entire length.
- Sequences with some limited regions of similarity will display short stretches of diagonal lines.
- Diagonals on either side of the main diagonal indicate repeat regions caused by duplication.

- A random scattering of dots reflects a lack of significant similarity. These dots are caused by short sub-sequences that match by chance alone.

For more information on dotplots, refer to the paper by Maizel & Lenk [14].

4.4 Sequence Alignments

Over evolutionary time, related DNA or amino acid sequences diverge through the accumulation of mutation events such as nucleotide or amino acid substitutions, insertions and deletions.

A *sequence alignment* is an attempt to determine regions of homology in a set of sequences. It consists of a table with one sequence per row, and with each column containing homologous residues from the different sequences, e.g. residues that are thought to have evolved from a common ancestral nucleotide/amino acid. If it is thought that the ancestral nucleotide/amino acid got lost on the evolutionary path to one descendant sequence, this sequence will show a special gap character "–" in that alignment column.

4.4.1 Pairwise sequence alignments

There are two types of pairwise alignments: *local* and *global* alignments.

A Local Alignment. A local alignment is an alignment of two sub-regions of a pair of sequences [21]. This type of alignment is appropriate when aligning two segments of genomic DNA that may have local regions of similarity embedded in a background of a non-homologous sequence.

A Global Alignment. A global alignment is a sequence alignment over the entire length of two or more nucleic acid or protein sequences. In a global alignment, the sequences are assumed to be homologous along their entire length [16].

Scoring systems in pairwise alignments

In order to align a pair of sequences, a scoring system is required to score matches and mismatches. The scoring system can be as simple as "+1" for a match and "-1" for a mismatch between the pair of sequences at any given site of comparison. However substitutions, insertions and deletions occur at different rates over evolutionary time. This variation in rates is the result of a large number of factors, including the mutation process, genetic drift and natural selection. For protein sequences, the relative rates of different substitutions can be empirically determined by comparing a large number of related sequences. These empirical measurements can then form the basis of a scoring system for aligning subsequent sequences. Many scoring

systems have been developed in this way. These matrices incorporate the evolutionary preferences for certain substitutions over other kinds of substitutions in the form of log-odd scores. Popular matrices used for protein alignments are BLOSUM [10] and PAM [2] matrices.

Note: The BLOSUM and PAM matrices are substitution matrices. The number of a BLOSUM matrix indicates the threshold (%) similarity between the sequences originally used to create the matrix. BLOSUM matrices with higher numbers are more suitable for aligning closely related sequences. For PAM, the lower numbered tables are for closely related sequences and higher numbered PAMs are for more distant groups.

When aligning protein sequences in Geneious, a number of BLOSUM and PAM matrices are available.

Algorithms for pairwise alignments

Once a scoring system has been chosen, we need an algorithm to find the optimal alignment of two sequences. This is done by inserting gaps in order to maximize the alignment score. If the sequences are related along their entire sequence, a global alignment is appropriate. However, if the relatedness of the sequences is unknown or they are expected to share only small regions of similarity, (such as a common domain) then a local alignment is more appropriate.

An efficient algorithm for global alignment was described by Needleman and Wunsch [16], and their algorithms was later extended by Gotoh to model gaps more accurately [6]. For local alignments, the Smith-Waterman algorithm [21] is the most commonly used. See the references provided for further information on these algorithms.

Pairwise alignment in Geneious

A dotplot is a comparison of two sequences. A pairwise alignment is another such comparison with the aim of identifying which regions of two sequences are related by common ancestry and which regions of the sequences have been subjected to insertions, deletions, and substitutions.

The options available for the alignment cost matrix will depend on the kind of sequence.

- Protein sequences have a choice of PAM [2] and BLOSUM [10] matrices.
- Nucleotide sequences have choices for a pair of match/mismatch costs. Some scores distinguish between two types of mismatches: transition and transversion. Transitions ($A \leftrightarrow G$, $C \leftrightarrow T$) generally occur more frequently than transversions. Differences in the ratio of transversions and transversions result in various models of substitution. When applicable, Geneious indicates the target sequence similarity for the alignment scores, i.e. the amount of similarity between the sequences for which those scores are optimal.

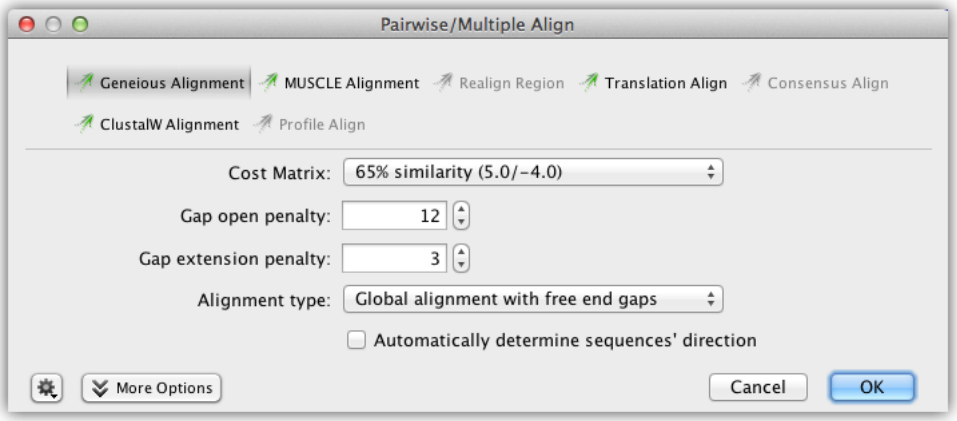


Figure 4.1: Options for nucleotide pairwise alignment

- Both protein and nucleotide pairwise alignments have choices for gap open / gap extension penalties/costs. Unlike many alignment programs these values are not restricted to integers in Geneious.

The score of a pairwise alignment is:

$matchCount \times matchCost + mismatchCount \times mismatchCost$

For each gap of length n, a score of $gapOpenPenalty + (n - 1) \times gapExtensionPenalty$ is subtracted from this.

Where

- $gapOpenPenalty =$ The "gap open penalty" setting in Geneious.
- $gapExtensionPenalty =$ The "gap extension penalty" setting in Geneious.
- $matchCost =$ The first number in the Geneious cost matrix.
- $mismatchCost =$ The second number in the Geneious cost matrix.
- $matchCount =$ The number of matching residues in the alignment.
- $mismatchCount =$ The number of mismatched residues in the alignment.

When doing a *Global alignment with free end gaps*, gaps at either end of the alignment are not penalized when determining the optimal alignment. This is especially useful if you are aligning sequence fragments that overlap slightly in their starting and ending positions, e.g. when

using two slightly different primer pairs to extract related sequence fragments from different samples. You can also do a *Local Alignment* if you want to allow free end overlaps, rather than just free end gaps in one alignment.

If you are aligning nucleotide sequences, you will also have the option of doing your alignment by translation and back. To view the options for translation alignment, click the 'More Options' button that the bottom of the alignment dialog. The translation alignment options will appear. Here you can set the genetic code and translation frame for the translation as well as the cost matrix, gap open penalty and gap extension penalty for the alignment. If you want to set the alignment type (global or local) or choose to automatically determine the sequences' direction, do it in the main section of the dialog.

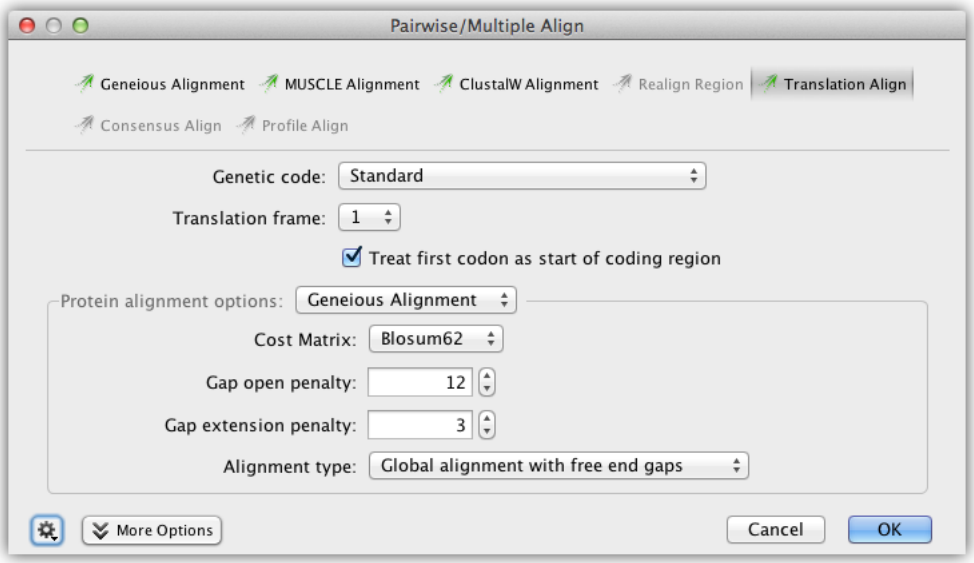


Figure 4.2: Options for nucleotide translation alignment

4.4.2 Multiple sequence alignments

A multiple sequence alignment is a comparison of multiple related DNA or amino acid sequences. A multiple sequence alignment can be used for many purposes including inferring the presence of ancestral relationships between the sequences. It should be noted that protein sequences that are structurally very similar can be evolutionarily distant. This is referred to as distant homology. While handling protein sequences, it is important to be able to tell what a multiple sequence alignment means – both structurally and evolutionarily. It is not always possible to clearly identify structurally or evolutionarily homologous positions and create a single "correct" multiple sequence alignment [3].

Multiple sequence alignments can be done by hand but this requires expert knowledge of molecular sequence evolution and experience in the field. Hence the need for automatic multiple sequence alignments based on objective criteria. One way to score such an alignment would be to use a probabilistic model of sequence evolution and select the alignment that is most probable given the model of evolution. While this is an attractive option there are no efficient algorithms for doing this currently available. However a number of useful heuristic algorithms for multiple sequence alignment do exist.

Progressive pairwise alignment methods

The most popular and time-efficient method of multiple sequence alignment is progressive pairwise alignment. The idea is very simple. At each step, a pairwise alignment is performed. In the first step, two sequences are selected and aligned. The pairwise alignment is added to the mix and the two sequences are removed. In subsequent steps, one of three things can happen:

- Another pair of sequences is aligned
- A sequence is aligned with one of the intermediate alignments
- A pair of intermediate alignments is aligned

This process is repeated until a single alignment containing all of the sequences remains. Feng & Doolittle were the first to describe progressive pairwise alignment [5]. Their algorithm used a guide tree to choose which pair of sequences/alignments to align at each step. Many variations of the progressive pairwise alignment algorithm exist, including the one used in the popular alignment software ClustalX [23].

Multiple sequence alignment in Geneious

Multiple sequence alignment in Geneious is done using progressive pairwise alignment. The neighbor-joining method of tree building is used to create the guide tree.

As progressive pairwise alignment proceeds via a series of pairwise alignments this function in Geneious has all the standard pairwise alignment options. In addition, Geneious also has the option of refining the multiple sequence alignment once it is done. “Refining” an alignment involves removing sequences from the alignment one at a time, and then realigning the removed sequence to a “profile” of the remaining sequences. The number of times each sequence is realigned is determined by the “refinement iterations” option in the multiple alignment window. The resulting alignment is placed in the folder containing the sequences aligned.

A profile is a matrix of numbers representing the proportion of symbols (nucleotide or amino acid) at each position in an alignment. This can then be pairwise aligned to another sequence

or alignment profile. When pairwise aligning profiles, mismatch costs are weighted proportional to the fraction of mismatching bases and gap introduction and gap extension costs are proportionally reduced at sites where the other profile contains some gaps.

In some cases building a guide tree can take a long time since it requires making a pairwise alignment between each pair of sequences. The "build guide tree via alignment" option may speed this part by taking a different route. First make a progressive multiple alignment using a random ordering, and use that alignment to build the guide tree. Notice that while this typically speeds up the process that may not be the case when the sequences are very distant genetically.

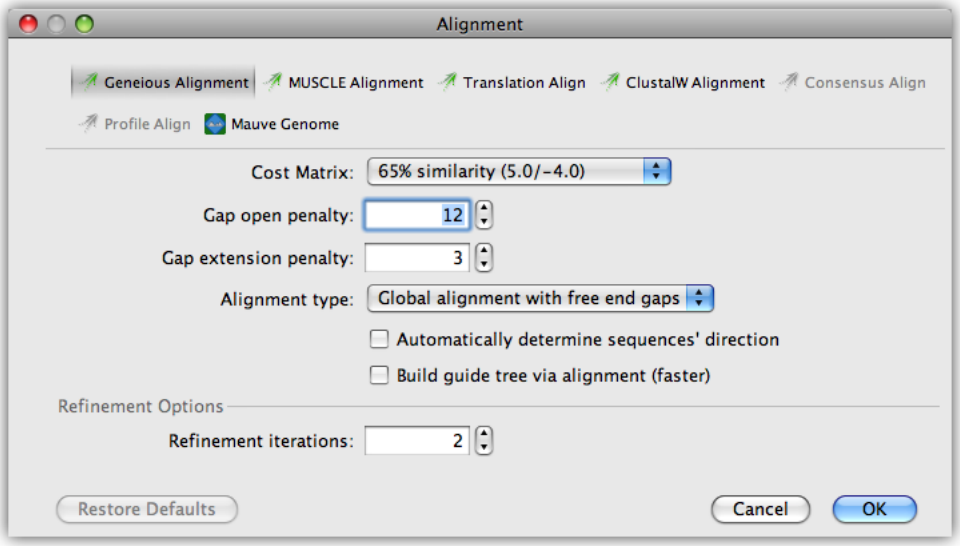


Figure 4.3: The multiple alignment window

You can also do a multiple alignment via translation and back, as with pairwise alignment.

4.4.3 Sequence alignment using ClustalW

ClustalW is a widely used program for performing sequence alignment [24, 23]. Geneious allows you to run ClustalW directly from inside the program without having to export or import your sequences.

If you do not have ClustalW or are unsure if you do, you should attempt to perform a ClustalW alignment without specifying a location. Geneious will then present you with options including details on how to download ClustalW, and will offer to automatically search for ClustalW on your hard drive.

To perform an alignment using ClustalW, select the sequences or alignment you wish to align and select the "Align/Assemble" button from the Toolbar and choose "Multiple Alignment." At the top of the alignment options window, there are buttons allowing you to select the type of alignment you wish to do. Choose "ClustalW" here, and the options available for a ClustalW alignment will be displayed.

The options are:

- *ClustalW Location:* This should be set to the location of the ClustalW program on your computer. Enter the path to it in the text field or click the 'Browse' button to browse for the location. If the location is invalid and you attempt to perform an alignment Geneious will tell you and offer the options detailed above for getting or finding ClustalW.
- *Cost Matrix:* Use this to select the desired cost matrix for the alignment. The available options here will change according to the type of the sequences you wish to align. You can also click the 'Custom File' button to use a cost matrix that you have on your computer (the format of these is the same as for the program BLAST).
- *Gap open cost* and *Gap extend cost:* Enter the desired gap costs for the alignment.
- *Free end gaps:* Select this option to avoid penalizing gaps at either end of the alignment. See details in the Pairwise Alignment section above.
- *Preserve original sequence order:* Select this option to have the order of the sequences in the table preserved so that the alignment contains the sequences in the same order.
- *Additional options:* Any additional parameters accepted by the ClustalW command line program can be entered here. Refer to the ClustalW manual for a description of the available parameters.

You can also do a clustal alignment via translation and back, as with pairwise alignment.

After entering the desired options click 'OK' and ClustalW will be called to align the selected sequences or alignment. Once complete, a new alignment document will be generated with the result as detailed previously.

4.4.4 Sequence alignment using MUSCLE

MUSCLE is public domain multiple alignment software for protein and nucleotide sequences. MUSCLE stands for multiple sequence comparison by log-expectation. See `http://www.drive5.com/muscle/`.

To perform an alignment using MUSCLE, select the sequences or alignment you wish to align and select the "Align/Assemble" button from the Toolbar and choose "Multiple Alignment". At the top of the alignment options window ,there are buttons allowing you to select the type of

alignment you wish to do. Choose "MUSCLE" here, and the options available for a MUSCLE alignment will be displayed.

For more information on muscle and its options, please refer to the original documentation for the program: http://www.drive5.com/muscle/muscle.html.

4.4.5 Combining alignments and adding sequences to alignments

"Consensus Alignment" allows you to align two or more alignments together (and create a single alignment) and align a new sequence in to an existing alignment. Select the sequences or alignment you wish to align and select the "Align/Assemble" button from the Toolbar and choose "Multiple Alignment." Consensus alignment allows you to choose which alignment algorithm to use for aligning the consensus sequences. All of the pairwise and multiple alignment algorithms are available. The consensus sequence used for each alignment is a 100% consensus with gaps ignored.

4.5 Building Phylogenetic trees

Geneious provides some basic phylogenetic tree reconstruction algorithms for a preliminary investigation of relationships between newly acquired sequences. For more sophisticated methods of phylogenetic reconstruction such as Maximum Likelihood and Bayesian MCMC we recommend specialist software such as MrBayes [19] and PhyML [7] which are available as a plugins to Geneious. These can be downloaded from the plugins page on our website.

Geneious implements the Neighbor-joining [20] and UPGMA [15] methods of tree reconstruction.

4.5.1 Phylogenetic tree representation

A phylogenetic tree describes the evolutionary relationships amongst a set of sequences. They have a few commonly associated terms that are depicted in Figure 3.12 and are described below.

Branch length. A measure of the amount of divergence between two nodes in the tree. Branch lengths are usually expressed in units of substitutions per site of the sequence alignment.

Nodes or internal nodes of a tree represent the inferred common ancestors of the sequences that are grouped under them.

Tips or leaves of a tree represent the sequences used to construct the tree.

Taxonomic units. These can be species, genes or individuals associated with the tips of the tree.

A phylogenetic tree can be rooted or unrooted. A rooted tree consists of a root, or the common ancestor for all the taxonomic units of the tree. An unrooted tree is one that does not show the position of the root. An unrooted tree can be rooted by adding an outgroup (a species that is distantly related to all the taxonomic units in the tree). A common format for representing phylogenetic trees is the Newick format [13].

4.5.2 Neighbor-joining

In this method, neighbors are defined as a pair of leaves with one node connecting them. The principle of this method is to find pairs of leaves that minimize the total branch length at each stage of clustering, starting with a star-like tree. The branch lengths and an unrooted tree topology can quickly be obtained by using this method without assuming a molecular clock [20].

4.5.3 UPGMA

This clustering method is based on the assumption of a molecular clock [15]. It is appropriate only for a quick and dirty analysis when a rooted tree is needed and the rate of evolution is does not vary much across the branches of the tree.

4.5.4 Distance models or molecular evolution models for DNA sequences

The evolutionary distance between two DNA sequences can be determined under the assumption of a particular model of nucleotide substitution. The parameters of the substitution model define a rate matrix that can be used to calculate the probability of evolving from one base to another in a given period of time. This section briefly discusses some of the substitution models available in Geneious. Most models are variations of two sets of parameters – the *equilibrium frequencies* and *relative substitution rates*.

Equilibrium frequencies refer to the background probability of each of the four bases A, C, G, T in the DNA sequences. This is represented as a vector of four probabilities $\pi_A, \pi_C, \pi_G, \pi_T$ that sum to 1.

Relative substitution rates define the rate at which each of the transitions ($A \leftrightarrow G$, $C \leftrightarrow T$) and transversions ($A \leftrightarrow C$, $A \leftrightarrow T$, $C \leftrightarrow G$, $G \leftrightarrow T$) occur in an evolving sequence. It is represented as a 4x4 matrix with rates for substitutions from every base to every other base.

Additionally, *gaps* are not penalized when using the Geneious Tree Builder. Comparisons involving any gaps are ignored when calculating the distance matrix.

Jukes Cantor

This is the simplest substitution model [11]. It assumes that all bases have the same equilibrium base frequency, i.e. each nucleotide base occurs with a frequency of 25% in DNA sequences and each amino acid occurs with a frequency of 5% in protein sequences. This model also assumes that all nucleotide substitutions occur at equal rates and all amino acid replacements occur at equal rates.

HKY

The HKY model [9] assumes every base has a different equilibrium base frequency, and also assumes that transitions evolve at a different rate to the transversions.

Tamura-Nei

This model also assumes different equilibrium base frequencies. In addition to distinguishing between transitions and transversions, it also allows the two types of transitions ($A \leftrightarrow G$ and $C \leftrightarrow T$) to have different rates [22].

4.5.5 Resampling – Bootstrapping and jackknifing

Resampling is a statistical technique where a procedure (such as phylogenetic tree building) is repeated on a series of data sets generated by sampling from one original data set. The results of analyzing the sampled data sets are then combined to generate summary information about the original data set.

In the context of tree building, resampling involves generating a series of sequence alignments by sampling columns from the original sequence alignment. Each of these alignments (known as pseudoreplicates) is then used to build an individual phylogenetic tree. A consensus tree can then be constructed by combining information from the set of generated trees or the topologies that occur can be sorted by their frequency (see below). [4].

Bootstrapping is the statistical method of resampling with replacement. To apply bootstrapping in the context of tree building, each pseudo-replicate is constructed by randomly sampling columns of the original alignment with replacement until an alignment of the same size is obtained [4].

Jackknifing is a statistical method of numerical resampling based on deleting a portion of the original observations for each pseudo-replicate. A 50% jackknife randomly deletes half of the columns from the alignment to create each pseudo-replicate.

4.5.6 Consensus trees

A consensus tree provides an estimate for the level of support for each clade in the final tree. It is built by combining clades which occurred in at least a certain percentage of the resampled trees. This percentage is called the consensus support threshold. A 100% support threshold results in a *"Strict consensus tree"* which is a tree where the included clades are those that are present in all the trees of the original set. A 50% threshold results in a *"Majority rule consensus tree"* that includes only those clades that are present in the majority of the trees in the original set. A threshold less that 50% gives rise to a *"Greedy consensus tree"*. In constructing a *"Greedy consensus tree"* clades are first ordered according to the number of times they appear (i.e. the amount of support they have), then the consensus tree is constructed progressively to include all those clades whose support is above the threshold **and** that are compatible with the tree constructed so far.

The length of the consensus tree branches is computed from the average over all trees containing the clade. The lengths of tip branches are computed by averaging over all trees.

Note: The above definitions apply to rooted trees. The same principles can be applied to unrooted trees by replacing "clades" with "splits". Each branch (edge) in an unrooted tree corresponds to a different split of the taxa that label the leaves of this tree.

4.5.7 Sort topologies

This will produce one or more trees summarizing the results of resampling. The frequency of each topology in the set of original trees is calculated and the topologies are sorted by their frequency. A number of these topologies, based on the topology threshold, will be output as summary trees. The summary trees have branch lengths that are the average of the lengths of the same branch from trees with the same topology.

The topology threshold determines what percentage of the original tree topologies must be represented by the summarizing topologies. The most common topology will always be output as the first summary tree. If the frequency (%) of this does not meet the threshold then the next most frequent topology will be added, and so on until the total frequency of the topologies reaches the threshold value.

A topology threshold of 0 will result in only the most common topology being output, a threshold of 100 will result in all topologies being output.

4.5.8 Tree building in Geneious

Geneious can build a phylogenetic tree for a set of sequences using pairwise genetic distances. To build a tree, select an alignment or a set of related sequences (all DNA or all protein) in the Document table and click the "Tree" icon or choose this option from the Tools menu.

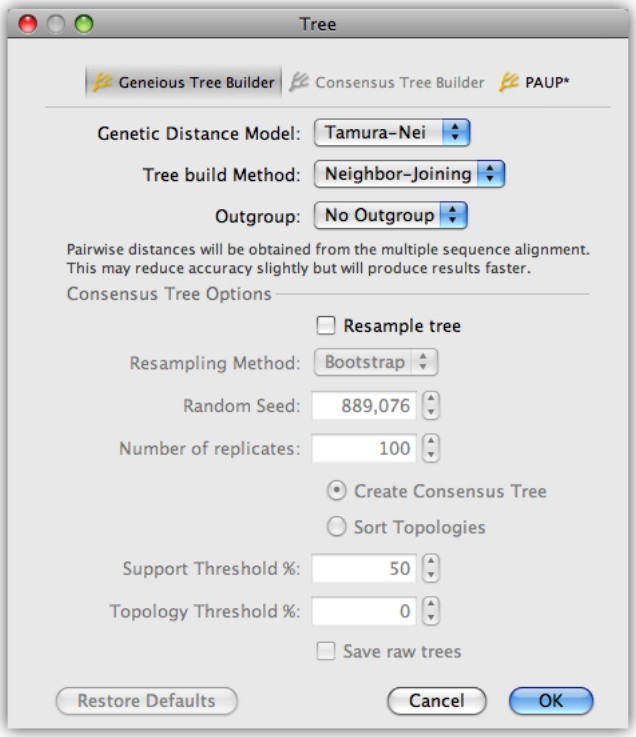


Figure 4.4: Tree building options in Geneious

Tree building from an alignment

If you are building a tree from an alignment, the following options are seen in the tree window.

If you select a tree document (which contains an alignment) then the alignment will simply be extracted from the tree and used in the tree building process.

- *Genetic distance model*. This lets the user choose the kind of substitution model used to estimate branch lengths. If you are building a tree from DNA sequences you have the choices "Jukes Cantor", "HKY" and "Tamura Nei". If you are building a tree from amino acid sequences you only have the option of "Jukes Cantor" distance correction.
- *Tree building method*. There are two methods under this option – Neighbor joining [20] and UPGMA [15].
- *Create consensus via resampling*. Check this box to build a consensus tree using resampling of sequence alignment data.
- *Resample tree* Check this to perform resampling.
- *Resampling method*. Either bootstrapping or jackknifing can be performed when resampling columns of the sequence alignment.
- *Number of samples*. The number of alignments and trees to generate while resampling. A value of at least 100 is recommended.
- *Create Consensus Tree*. Choose this to create a consensus tree from the samples.
- *Sort Topologies*. Produce trees which summarise the topologies resulting from resampling. See above for more details.
- *Support threshold*. This is used to decide which monophyletic clades to include in the consensus tree, after comparing all the trees in the original set. (see Consensus Tree section above)
- *Topology Threshold*. The percentage of topologies in the original trees which must be represented by the summarizing topologies.
- *Save raw trees*. If this is turned on then all of the trees created during resampling will be save in the resulting tree document. The number of raw trees saved will therefore be equal to the number of samples.

Creating a consensus tree of existing trees

If you select a tree set document and choose "Tree" then the Consensus option will be available at the to of the tree builder options. This will create a consensus tree using the trees already

in the document (no resampling will be performed) and it will either be added to the tree document or saved as a separate tree document.

The only option available here is the consensus support threshold.

4.6 PCR Primers

Geneious provides several operations for designing and working with PCR Primers and DNA or hybridisation probes. PCR Primers and DNA or hybridisation probes can be designed for or tested on existing nucleotide sequences or alignments. A PCR product can be extracted from a sequence that has been annotated with both a forward and a reverse primer. 5′ extensions consisting of restriction enzymes or arbitrary sequence may also be added to primer documents.

In addition Geneious can determine the primer characteristics for a primer sized sequence and convert it into a primer. Characteristics can also be determined for any number of primer sized selections made in the Sequence View.

To use any one of these primer operations simply select the appropriate nucleotide sequences and either select "Primers" from the Tools menu or right-click (Ctrl+click on Mac OS X) on the document(s) and select "Primers". A popup menu will appear showing the operations valid for your current selection.

4.6.1 Design New Primers

Geneious uses Primer3 to design PCR primers. The Primer Design dialog allows you to set options for where your PCR primers should sit, what size product to return and characteristics such as primer length and melting temperature.

Task

Two tasks are available, "Design New" or "Design with Existing". "Design New" designs a pair of forward and reverse primers. You can specify if you wish to design with or without a matching probe. "Design with Existing" can design a partner primer to match an existing one, for example a reverse primer for a forward or vice versa. It also allows you to design a probe to match a pair of primers.

If any documents were selected which either are primer sequences or contain primer annotations then these will be made available for selection as primers in a drop-down box. Selected sequences are treated as primer or probe sequences if they are 150bp in length or less.

For each of these tasks, Generic or Cloning primers can be designed.

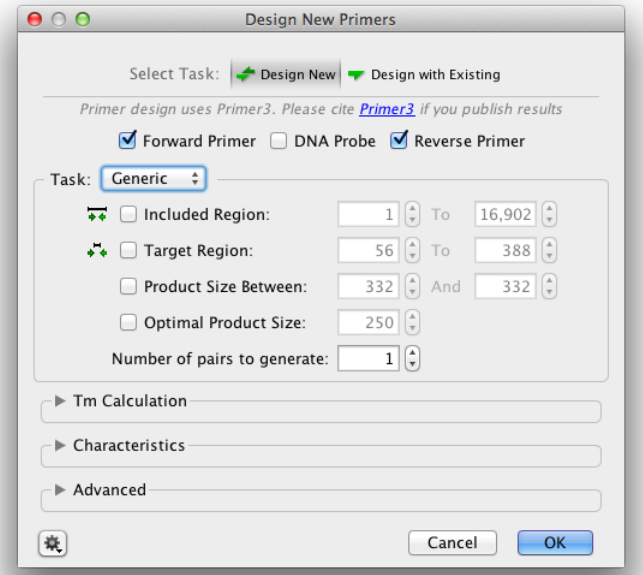


Figure 4.5: The primer design dialog

Generic primers

This option will design standard PCR primers according to the region input options you select. These options allow you to specify what part of a sequence you wish to amplify. Most options are optional and can be enabled or disabled with the associated check boxes beside them. If you have selected a region in the sequence before opening the primer dialog then this region will automatically be used for Included Region and Target Region. All of these are expressed in base pairs from the beginning of the sequence and are as follows:

- **Included Region:** Specifies the region of the sequence within which primers are allowed to fall. This must surround the target region and allows you to choose a small region on either side of the target in which primers must lie.
- **Target Region:** Specifies which region of the sequence you wish to amplify and unless the advanced options allow otherwise, the forward and reverse primers must fall somewhere outside this region.
- **Product Size:** Specifies the range of sizes which the product of a primer pair can have. The product size is the distance in bp between the beginning of the forward primer to the end of the reverse primer.

- **Optimal Product Size:** Specifies the preferred size of the product. Setting this will mean primer pairs that have a product size close to this will be chosen over those that do not. Warning: Setting these options can cause the primer design process to take considerably longer to complete.

The final option in this section is **Number of Pairs to Generate** which specifies how many candidate pairs of primers and DNA probes to generate and is compulsory. Setting this to 1 will give you only the primer pair which was considered best by the set parameters.

Cloning primers

This option allows you to design primers to amplify a specific region. Only the included region can be set, and the primers will be designed to the very ends of this region so that the entire region is included in the PCR product. This option is useful for amplifying an entire CDS for creating an insert for cloning.

Tm calculation

This section gives the formulas used to calculate the melting point of oligos. Under **Formula** you can choose between two different tables of thermodynamic parameters and methods for melting temperature calculation:

- **Breslauer et al. 1986** (http://www.pnas.org/content/83/11/3746). This is used by old versions of Primer3 (until version 1.0.1), and uses the formula for melting temperature calculation suggested by Rychlik et al. 1990.
- **SantaLucia 1998** (http://www.pnas.org/content/95/4/1460). This is the recommended value.

Three different Salt Correction Formula options are available:

- **Schildkraut and Lifson 1965** (http://dx.doi.org/10.1002/bip.360030207). This is used by old versions of Primer3 (until version 1.0.1)
- **SantaLucia 1998** (http://www.pnas.org/content/95/4/1460). This is the recommended value.
- **Owczarzy et al. 2004**, (http://dx.doi.org/10.1021/bi034621r)

Characteristics

The Characteristics section allows you to set absolute limits on properties of primers and probes such as melting point and GC content. Optimum values can also be specified. For details on individual options hover your mouse over them and a popup box will describe the function of the option.

Characteristics can be set for either **Primers** or **DNA Probes**, depending on the task you have chosen. The "Primer" section is available if one of "Forward Primer" or "Reverse Primer" is being designed or tested and "DNA Probe" is available if "DNA Probe" is being designed or tested. These two sections are quite similar; the DNA probe section has a subset of the options available in the primer section. This is because primers are usually chosen in pairs and so several options can be set for how pairs are chosen.

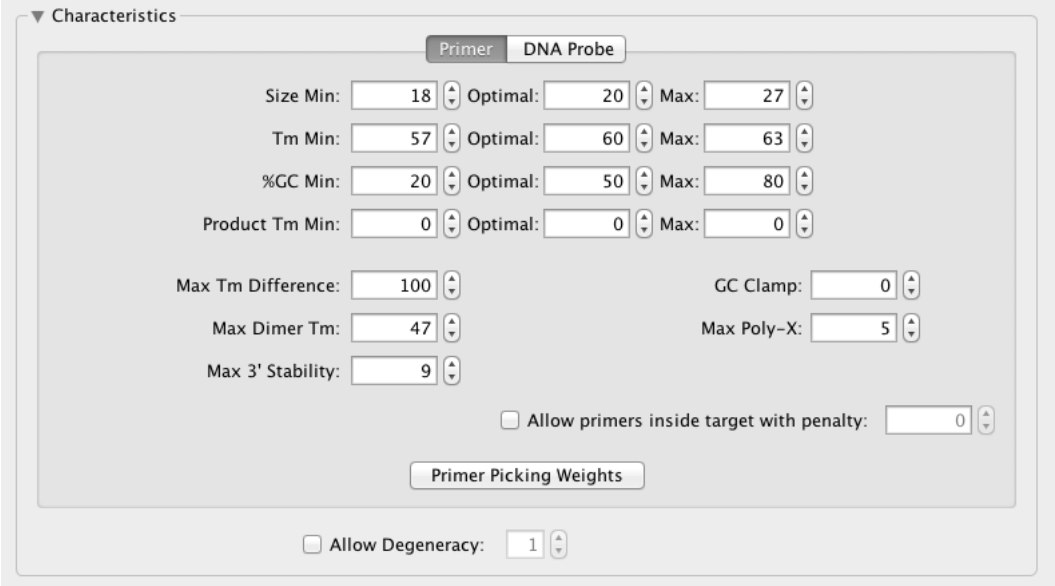

Characteristics
Primer
DNA Probe
Size Min: 18 Optimal: 20 Max: 27
Tm Min: 57 Optimal: 60 Max: 63
%GC Min: 20 Optimal: 50 Max: 80
Product Tm Min: 0 Optimal: 0 Max: 0
Max Tm Difference: 100
Max Dimer Tm: 47
Max 3' Stability: 9
GC Clamp: 0
Max Poly-X: 5
Allow primers inside target with penalty: 0
Primer Picking Weights
Allow Degeneracy: 1

Figure 4.6: Primer characteristics options

Primer Picking Weights

At the bottom of the Characteristics panel there is a "Primer Picking Weights" button. Clicking this brings up a second dialog containing many more options. The purpose of all of these options is to allow you to assign penalty weights to each of the parameters you can set in the options. The weight specified here determines how much of a penalty primers and probes get when they do not match the optimal options. The higher the value the less likely a primer or probe will be chosen if it does not meet the optimal value.

Some of the weights allow you to specify a "Less Than" and "Greater Than". This is for options which allow you to specify an optimum score such as GC content. These weights are used when looking at primers whose value for this option falls below and above the optimum respectively. The other weights are applied no matter in which direction they vary.

For details on individual options in the Primer Picking Weights dialog, again hover your mouse over the option to see a short description.

Degenerate Primer Design

A degenerate primer contains a mix of bases at one or more sites. They are useful when you only have the protein sequence of your gene of interest so want to allow for the degeneracy in the genetic code, or when you want to isolate similar genes from a variety of species where the primer binding sites may not be identical. You can design degenerate primers in Geneious by using either a sequence containing ambiguous bases or an alignment as the template and checking the **Allow degeneracy** box. The degeneracy value that you specify is the maximum number of primers that any primer sequence is allowed to represent. For example, a primer which contains the nucleotide character N once (and no other ambiguities) has a degeneracy of 4 because N represents the four bases A,C,G and T. A primer that contains an N and an R has degeneracy $4 \times 2 = 8$ because R represents the two bases A and G.

Advanced Options

In the Advanced panel there are options to add 5′ extensions to primers and to specify a mispriming library.

A 5′ extension can be your own sequence, a restriction enzyme or Gateway site, or a combination of these. For more information see section 4.6.5.

A mispriming library is a set of sequences (usually repeats) which the primers should not bind to. Four inbuilt libraries are available for selection, or you can upload a custom library of sequences in fasta format. For more information on the inbuilt libraries, see the Primer3 help page.

Output from Primer Design

Once the task and options have been set, click the 'OK' button to design the primers. A progress bar may appear for a short time while the process completes. When complete each of the sequences will have the designed primers and probes added to them as sequence annotations. If you are designing primers off an alignment the primer will be annotated on the consensus sequence. The annotations will be labelled with the base number the primer starts at, followed

by either F (forward primer), R (reverse primer), or P (probe). Primers will be coloured green and probes red.

Detailed information such as melting point, tendency to form primer-dimers and GC content can be seen by hovering the mouse over the primer annotation. The information will be presented in a popup box. Alternatively, double clicking on an annotation will display its details in the annotation editing dialog. Table 4.1 shows how the values in the Geneious primer annotation map to the original Primer3 values.

The best way to save a primer or DNA probe for further testing or use is to select the annotation for that primer and click the 'Extract' button in the sequence viewer. This will generate a separate, short sequence document which just contains the primer sequence and the annotation (so it retains all the information on the primer). In the case of the reverse primer it will automatically be reverse complemented.

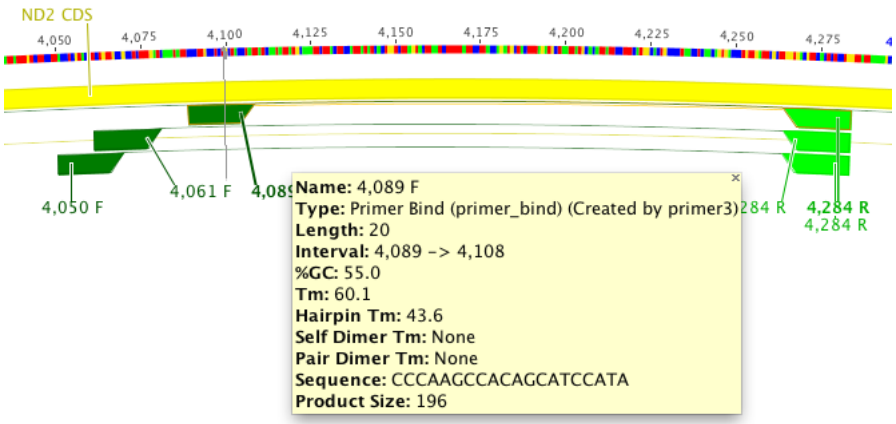


Figure 4.7: Primer design output

When no primers can be found

If no primers or DNA probes that match the specified criteria can be found in one or more of the sequences then a dialog is shown describing how many had no matches and for what reasons.

To see why no primers or DNA probes were found for particular sequences, click the 'Details' button at the bottom of the dialog. The dialog will then open out to display a list of all the sequences for which no primers or DNA probes were found. For each of the sequences the following information is listed:

Geneious Primer Characteristics	Primer3 Web Interface	Primer3 Command Line
%GC	Primer GC%	PRIMER_{LEFT,RIGHT}_GC_PERCENT
Tm	Primer Tm	PRIMER_{LEFT,RIGHT}_TM
Hairpin	Max Self Complementarity (Any)	PRIMER_{LEFT,RIGHT,INTERNAL_OLIGO}_SELF_ANY
Primer-Dimer	Max 3′ Self Complementarity	PRIMER_{LEFT,RIGHT,INTERNAL_OLIGO}_SELF_END
Monovalent Salt Concentration	Concentration of monovalent cations	PRIMER_SALT_CONC
Divalent Salt Concentration	Concentration of divalent cations	PRIMER_DIVALENT_CONC
DNTP Concentration	Concentration of dNTPs	PRIMER_DNTP_CONC
Sequence	Seq	PRIMER_{LEFT,RIGHT}_SEQUENCE
Product Size	Product Size Ranges	PRIMER_PRODUCT_SIZE
Pair Hairpin	PAIR ANY COMPL	PRIMER_PAIR_COMPL_ANY
Pair Primer-Dimer	PAIR 3′ COMPL	PRIMER_PAIR_COMPL_END
Pair Tm Diff	Max Tm Difference	PRIMER_PRODUCT_TM_OLIGO_TM_DIFF

Table 4.1: Geneious primer characteristics and their Primer3 counterparts

- Which of Forward Primer, Reverse Primer, Primer Pair and/or DNA Probe could not be found in the sequence
- For each of these, specific reasons for rejection are listed (eg. “Tm too high” or “Unacceptable product size”) along with a percentage which expresses how many of the candidate primers or probes were rejected for this reason.

After examining the details you can choose take no action or continue and annotate the primer and/or DNA probes on the sequences which were successfully designed for.

4.6.2 Primer Database

The Primer Database consists of all the oligonucleotide documents that exist in your Local or Shared Databases. The “oligonucleotide” document type is a short nucleotide sequence representing either a primer or a probe. The text view lists the primer characteristics (Tm, GC *etc*). These properties can be shown in the document table. Tm is shown by default, but you can turn on others by right clicking on the table header.

Oligo sequences are created via one of the following methods:

- Extract a primer/probe annotation from a sequence
- Select “Sequence” → “New Sequence” from the menu and choose Primer or Probe as the type of the new sequence
- Select one or more existing primer sequences (maybe ones imported from a file) then click “Primers” → “Convert to Oligo” to transform them into oligo type sequences

If you select a target sequence and go to “Test with Saved Primers” or “Design Primers” → “Design With Existing”, Geneious will find all oligo sequences in your database and offer them

as options in the list of oligo sequences with no need to select them along with the target sequence before starting the operation.

The meta-data type "Primer Info" can be used to note the fridge location *etc* of a particular primer.

4.6.3 Test with Saved Primers

Primers and probes can also be quickly tested against large numbers of sequences to see which ones the primers will bind to. To test primers select the target sequences you want to test for compatibility with primers and choose the same "Primers" action from the menu and go to "Test with Saved Primers" in the popup menu that appears.

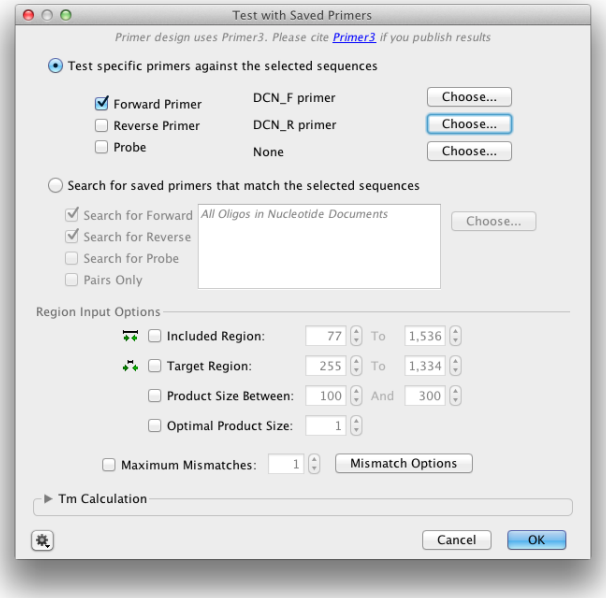


Figure 4.8: The primer test dialog

There are two ways in which Geneious can test your selection of primers and probes. The first option in the dialog tests a specific forward and reverse primer pair and/or probe. Clicking the **Choose** buttons next to forward, reverse and probe options will bring up your primer database, allowing you to select any primer in your database for testing.

The second option allows you to specify multiple primers and probes to test all selected sequences against. Click the Choose button then hold down CTRL-click (⌘-click on Mac) to select multiple primers and probes from many different locations in your database. Alternatively, you can select one or more folders to test with all the primers and probes inside them,

or click the Use All button to use every primer in your database.

As with the first option, you can choose which types of primers you'd like to test for, by selecting the checkboxes on the left. Note that each primer you select will be considered in both the forward and reverse roles, if you have checked both Search for Forward and Search for Reverse. One final checkbox, Pairs Only, forces primers and probes to be considered as pairs (with the probe inside), otherwise they can be found anywhere in the sequence with no constraints.

All of the same options available for designing primers also apply to testing so if the primers are expected to bind to quite different regions of the test sequences the primer binding region may have to be extended and the target region option can be omitted.

By default, only primers that match the target sequence exactly will be found. If you wish to allow a limited number of mismatches between the primer and target sequence you can specify this under **Maximum Mismatches**. You can limit the position in which mismatches are allowed by clicking the 'Mismatch Options' button.

Click the 'OK' button and testing will commence. Once complete, a dialog will present the results. This dialog tells you how many of the sequences were compatible with the specified primers and probes and provides details and choices very similar to the one described in section 4.6.1. The compatible primers can be annotated onto the sequences in a similar manner to that when designing primers. Additionally if the primer sequences were not already annotated with a primer annotation they will be annotated during testing.

4.6.4 Primer Characteristics

Characteristics for Selection

Primer Characteristics can also be determined on a selection in a larger sequence. Select a region of 150bp or less in the Sequence View and choose "Characteristics for Selection". The primer characteristics will then be added as an annotation over the exact region that was selected. This will also work on multiple selected regions in the Sequence View. Hold the Ctrl key while clicking and dragging to select multiple regions simultaneously.

Convert to Oligo

Geneious can convert any number of sequences that are 150 base pairs or fewer in length into primers. This operation will also determine the primer characteristics of the sequences, such as melting point. To do this, select your sequences and choose the same "Primers" action as you do with design or test, then choose "Convert to Oligo" from the popup menu that appears. If you select just two sequences you have the additional option of determining their pair characteristics. Determining the pair characteristics of two primer sequences can be used to see if two sequences can pair and how well they do so.

4.6.5 Primer Extensions

You can add a primer extension to an existing "oligonucleotide" sequence by selecting "Primers" → "Add 5′ Extension". You can add your own sequence, a restriction site, and/or Gateway cloning site. Multiple extensions can be added in one go, and the preview window in the 5′ extension dialog box shows how these extensions will be arranged on the primer. The order of 5′ additions can be edited by dragging and dropping them in this window.

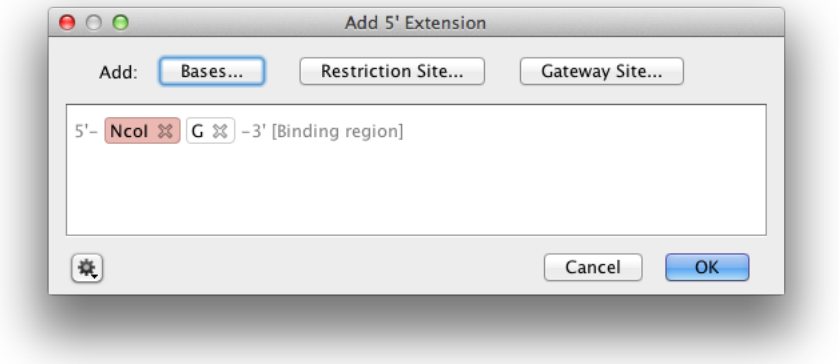


Figure 4.9: Adding 5′ extensions to a primer

Once added, the 5′ extension is shown in bold on the primer sequence and is not covered by the primer annotation, as shown in Figure 4.10. These extensions will not change the binding region of the primer and will be ignored when primer testing is conducted against potential target sequences.

If the primer is annotated onto a sequence following testing, the extension sequence is shown in the list of the annotation's qualifiers. If the primer or a PCR product is extracted from this annotation, the result will include the extension.

4.6.6 Manual primer design

It is possible to create PCR primers by adding a primer annotation directly onto a sequence. This is especially useful for cloning applications as generally the primers must bind to a specified set of bases at the beginning and end of the gene to be cloned. To manually add a primer, select the region of sequence where you wish the primer to bind and click "Add Annotation". Make the annotation type "primer bind" and you will then see primer-specific options and characteristics as in Figure 4.11. Changing the primer binding site position in the Add annotation window will automatically update the primer sequence and characteristics. A 5′ extension can also be added directly onto a primer in this step by clicking the button next to "Extension". See section 4.6.5 for more information on adding 5′ extensions.

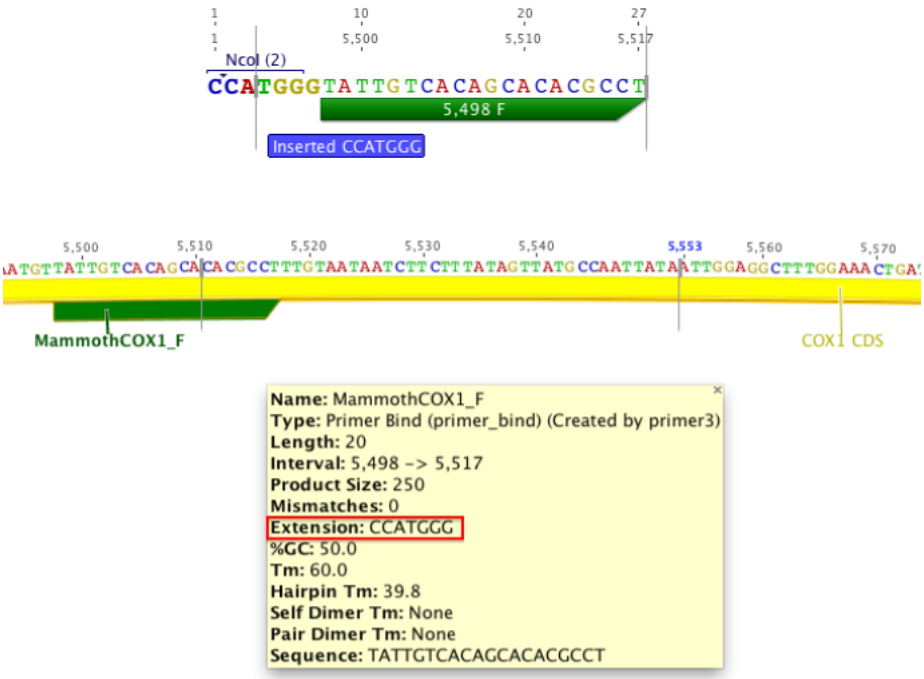


Figure 4.10: Primer annotation with 5′ extension

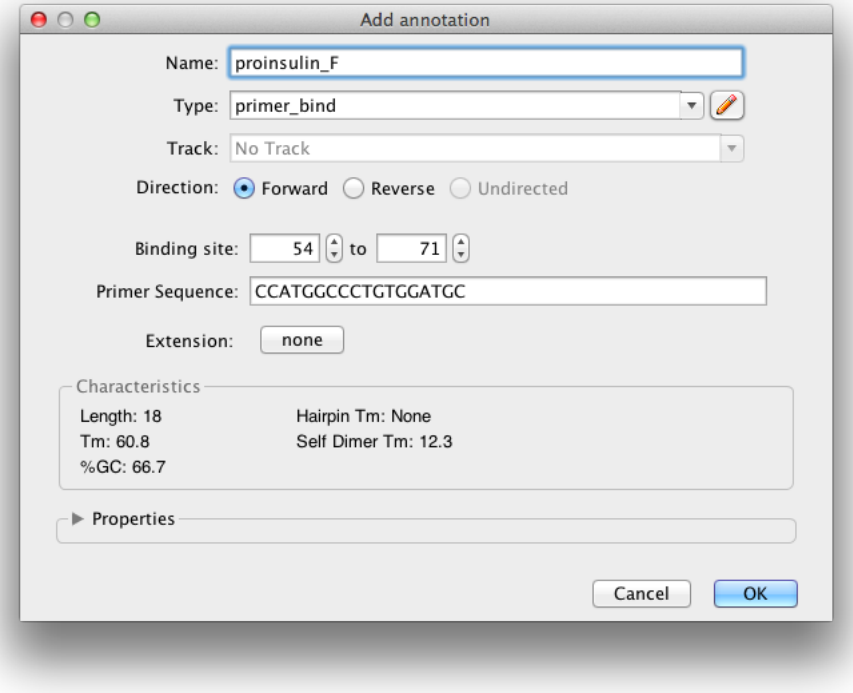


Figure 4.11: Create a primer by adding a primer annotation

4.6.7 Importing primers from a spreadsheet

You can import primers and probes directly into Geneious from Comma/Tab-Separated Values documents. You can either import them from the "Import" → "From File" menu, or simply paste the contents of the document into Geneious.

When Geneious has successfully recognized the file as CSV or TSV, you will see the following dialogue (Figure 4.12).

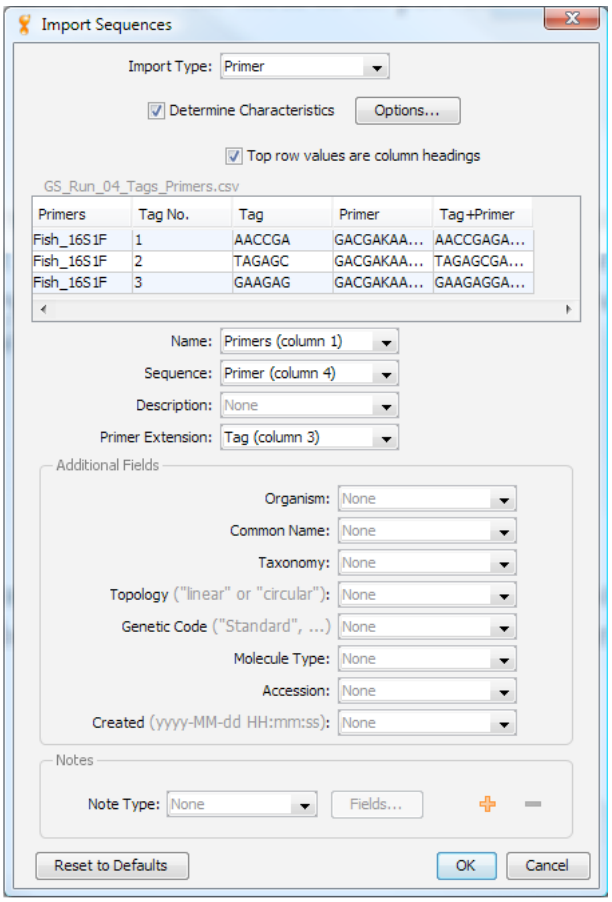


Figure 4.12: Importing primers from a spreadsheet

You will be asked which type of sequence you are importing. When you choose to import primers or probes, you will receive some options that allow you to determine characteristics for them as an extra step.

Immediately below this is a preview of the first few rows of data, and a checkbox that allows you to tell Geneious that the top row is a heading row and should be ignored.

Below the preview is a list of common and additional fields, along with dropdown boxes. These

boxes allow you to specify which column contains which piece of data – often, one or more of these won't be applicable and can be left as "None". Note that at minimum, you must specify a "Sequence" field.

Lastly, any additional data in the form of meta-data. Clicking the dropdown box next to 'Meta Data' at the bottom of the dialog will allow you to import values to meta-data, and clicking the $+$ or $-$ will allow you to insert or remove additional meta-data types. Next, click the "Fields..." button to bring up a dialog.

An additional set of dropdown boxes will allow you to specify again which columns of data contain the fields which comprise this meta-data type. This includes custom meta-data types that you have created and saved in the past.

When you're ready, hit "OK" to begin importing. When Geneious is done, you may be presented with the option of grouping the sequences you imported into a sequence list. This option is recommended if you're importing very large sets of sequences.

4.6.8 More Information

The Primer feature in Geneious is based on the program Primer3 http://bioinfo.ut.ee/primer3/.

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If you use the primer design feature of Geneious for publication we request that you cite primer3 as:

Steve Rozen and Helen J. Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386 Source code available at http://sourceforge.net/projects/primer3/.

Further information on the functionality of the primer design feature can be found in the primer3 documentation available here: http://primer3.ut.ee/primer3web_help.htm. Please note that some controls have been changed, renamed or removed from Geneious, but most of the primer3 functionality is available.

4.7 Contig Assembly

Contig assembly or sequence assembly is normally used to merge overlapping fragments of a DNA sequence into a contig which can be used to determine the original sequence. The contig essentially appears as a multiple sequence alignment of the fragments. After some manual

editing of the contig to resolve disagreements between fragments which result from read errors, the consensus sequence of the contig is extracted as the sequence being reconstructed.

Contig assembly is also used to align a large number of reads of the same sequence (from different individuals). This is done to find small differences between reads or SNPs (Single Nucleotide Polymorphisms). In this type of analysis the consensus sequence of the contig is not the interesting part, the differences between fragments is. This can also be done against a known reference sequence when differences between each of the fragments and the reference are of interest.

4.7.1 Assembling a Contig

To assemble a contig firstly select all of the sequences and/or contigs you wish to assemble along with the reference sequence (if you want to use one) in the document table then click "Align/Assemble" in the toolbar and choose "De Novo Assemble." The basic options for contig assembly will then be displayed.

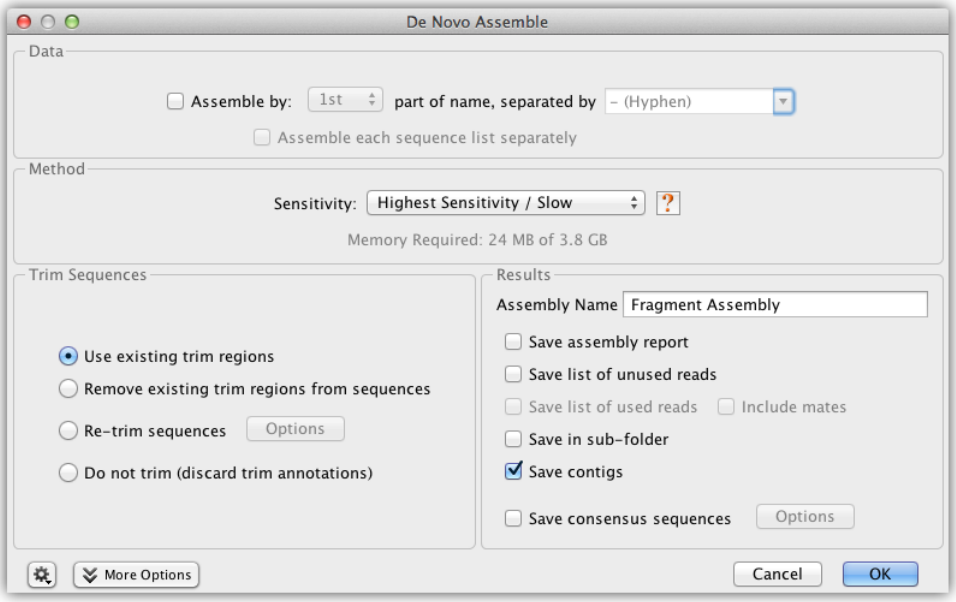


Figure 4.13: Basic de novo assembly options

The options available here are as follows:

- **Assemble by (aka Assemble by Name):** If you have selected several groups of fragments which are to be assembled separately, you can specify a delimiter and an index at which

the identifier can be found in all of the names. Sequences are grouped according to the identifier and each group is assembled separately. If a reference sequence is specified, it is used for all groups. eg. For the names A03.1.ab1, A03.2.ab1, B05.1.ab1, B05.2.ab1 etc where "A03" and "B05" are the identifiers you would choose "Assemble by 1st part of name, separated by . (full stop)"

- **Assembly method:** Specifies a trade off between the time it takes to assemble and the accuracy of the assembly. Higher sensitivity is likely to result in more reads being assembled.
- **Trim Sequences:** Select how to trim the ends of the sequences being assembled. See section 4.7.3.
- **Save assembly report:** Instead of displaying the results of the assembly in a dialog, the results are saved in a separate report document alongside the contig(s). This lists which fragments were successfully assembled and which contig they went in to along with a list of unassembled fragments.

Advanced Options (Click 'More Options')

- **Save results in a new subfolder named:** If selected, all results of the assembly will be saved to a new subfolder inside the one containing the fragments. This folder will always only contain the assembly results from the one most recent assembly - it creates a new folder each time it is run.
- **Alignment Options:** Penalties and scores used when aligning the fragments; these normally don't need to be changed.

Other advanced options depend on the assembly method selected. These are fully documented if you hover the mouse over them in Geneious. High Sensitivity/slowest assembly advanced options include

- **Minimum Overlap:** The minimum overlap (in nucleotides) between a sequence and any sequence in the contig required for the sequence to be included in the contig.
- **Overlap Identity:** The minimum identity (in percent) of the overlap region between a sequence and any sequence in the contig required for the sequence to be included in the contig.

Choose the options you require and click 'OK' to begin assembling the contig. Once complete, one or more contigs may be generated. If you got more contigs than you expect to get for the selected sequences then you should try adjusting the options for assembly. It is also possible that no contigs will be generated if no two of the selected sequences meet the overlap requirements.

Note: The orientation of fragments will be determined automatically, and they will be reverse complemented where necessary.

If you already have a contig and you want to add a sequence to it or join it to another contig then just select the contig and the contig/sequence and click assembly as normal.

Click 'More Options' in the assembly options to display the Alignment parameters. Here you can change the parameters used by Geneious when aligning fragments together. For sequences which are lower quality or contain many errors, the gap penalty should be decreased and the mismatch score should be increased.

The algorithm

The sequence assembler in Geneious is flexible enough to handle read errors consisting of either incorrect bases or short indels. It can handle data from any type of sequencing machine with reads of any length, including paired-reads and mixtures of reads from different sequencing machines (hybrid assemblies).

The de novo assembly algorithm used is a greedy algorithm which is similar to that used in multiple sequence alignment.

1. For each sequence a blast-like algorithm is used to find the closest matching sequence among all other sequences.
2. The highest scoring sequence and its closest matching sequence are merged together into a contig (reverse complementing if necessary). This process is repeated, appending sequences to contigs and joining contigs where necessary.
3. For paired read de novo assembly, 2 sequences with similar expected mate distances are given a higher matching score if their mates also score well against each other. Similarly a sequence and its mate will be given a higher score if they both align at approximately their expected distance apart to an already formed contig. The effect of this heuristic is that paired read de novo assembly starts out by finding 2 sets of paired reads and forming 2 contigs. Each of these 2 contigs will contain 1 sequence from each pair and the 2 contigs are expected to be separated by the expected mate distance. Assembly proceeds from there either adding new paired reads to the contigs or forming new pairs of contigs which eventually merge together. Due to the nature of this algorithm, paired read de novo assembly in Geneious only works well if you have high coverage of paired reads - a hybrid assembly of mostly unpaired data with a few paired reads will not make good use of the paired read data, but this is expected to improve in future versions.
4. Each contig generated by a gapped de novo assembly has some minor fine tuning performed on it both during assembly and upon completion. For each gapped position in a sequence, a base adjacent to the gap is shuffled along into the gap if it is the same base as the most common base in other sequences in the contig at that position. After doing this if any column now consists entirely of gaps that column is removed from the contig
5. Other minor heuristics are applied throughout the assembly to improve the results.

6. Both the Geneious de novo and reference assemblers use a deterministic method (even when spreading the work cross multiple CPUs) such that if you rerun the assembler using the same settings and same input data it will always produce the same results.

The reference assembly algorithm used is a seed and expand style mapper followed by an optional fine tuning step to better align reads around indels to each other rather than the reference sequence. Various optimizations and heuristics are applied at each stage, but a general outline of the algorithm is

1. First the reference sequence(s) is indexed to create a table making a record of all locations in the reference sequence that every possible word (series of bases of a specified length) occurs.

2. Each read is processed one at a time. Each word within that read is located in the reference sequence and that is used as a seed point where the matching range is later expanded outwards to the end of the read.

3. If a read does not find a perfectly matching seed, the assembler can optionally look for all seeds that differ by a single nucleotide.

4. Before the seed expansion step, all seeds for a single read that lie on the same diagonal are filtered down to a single seed.

5. During seed expansion, when mismatches occur a look-ahead is used decide whether to accept it as a mismatch or to introduce a gap (in either the reference sequence or read)

6. The mapper handles circular reference sequences by indexing reference sequence words spanning the origin and allowing the expansion step to wrap past the ends

7. All results are given a score based on the number of mismatches and gaps introduced. Normally the best scoring (or a random one of equally best scoring) matches are saved although there is an option to map the read to all best scoring locations

8. Paired reads are given an additional score penalty based on their distance from their expected distance so that they prefer mapping close to their expected distance with as few mismatches as possible, but they can also map any distance apart if an ideal location is not found.

9. The final optional fine tuning step at the end, shuffles the gaps around so that they reads better align to each other rather than the reference sequence.

For further details and for a comparison of the Geneious reference assembler to other software, see http://desktop-links.geneious.com/assets/documentation/geneious/GeneiousReadM
pdf.

4.7.2 Assembly to a reference sequence

Assembling to reference is used when you have known sequence and you wish to compare a number of reads of the same sequence with it to locate differences or SNPs. To perform assembly to a reference sequence select the sequences and the reference sequence and click "Align/Assemble" and choose "Map to Reference". Choose the name of the sequence you wish to use as the reference in the Align to reference option and click 'OK'. One contig will be produced at most and this will display the reference sequence at the top of the alignment view with all other sequences below it.

See section 4.7.6 for details on identifying differences or SNPs.

When aligning to reference the sequences are not aligned to each other in any way, each of them is instead aligned to the reference sequence independently and the pairwise alignments are combined into a contig. The high, medium and low sensitivity options perform a fine tuning step after the initial assembly to make reads which overlap from the initial assembly stage align better to each other.

If you just wish to use a reference sequence to help construction of the contig where the reads extend beyond the length of the reference then you have two options. With iterative fine tuning, reads can extend a bit further past the ends of the reference sequence on each iteration so make sure you set the number of iterations high enough. Or you could select all sequences including the reference and use the De Novo assembler.

4.7.3 Trimming

Trimming low quality ends of sequences is normally performed before assembling a contig. This is because the noise introduced by low quality regions and vector contamination can produce incorrect assemblies.

The easiest way to trim sequences is at the assembly step. Select the trim options you wish to use in the Assembly options and click 'OK'. The sequences will be trimmed and assembled in one operation. This means you cannot view the trimming that Geneious uses before assembly is performed, but the trimmed regions will still be available and adjustable after assembly is complete.

Trimmed annotations on the ends of sequences are ignored when calculating the consensus sequence for a contig. So although the trimmed regions are visible, they do not affect the results of assembly at all.

Sequence trimming can be performed before assembly by selecting the sequences you wish to trim and selecting "Annotate & Predict"→"Trim Ends". This will add "Trimmed" annotations to the sequences which are ignored in the construction of a contig. When performing "Assembly" from sequences which have been annotated in this way, select "Use Existing Trim Regions".

Trimmed annotations can also be created manually using the annotation editing in the sequence viewer. If you create annotations of type "trimmed" and save them then Geneious will treat them the same as ones generated automatically and they will be ignored during assembly. Trimmed annotations can also be modified in this way before or after assembly.

Trimming options

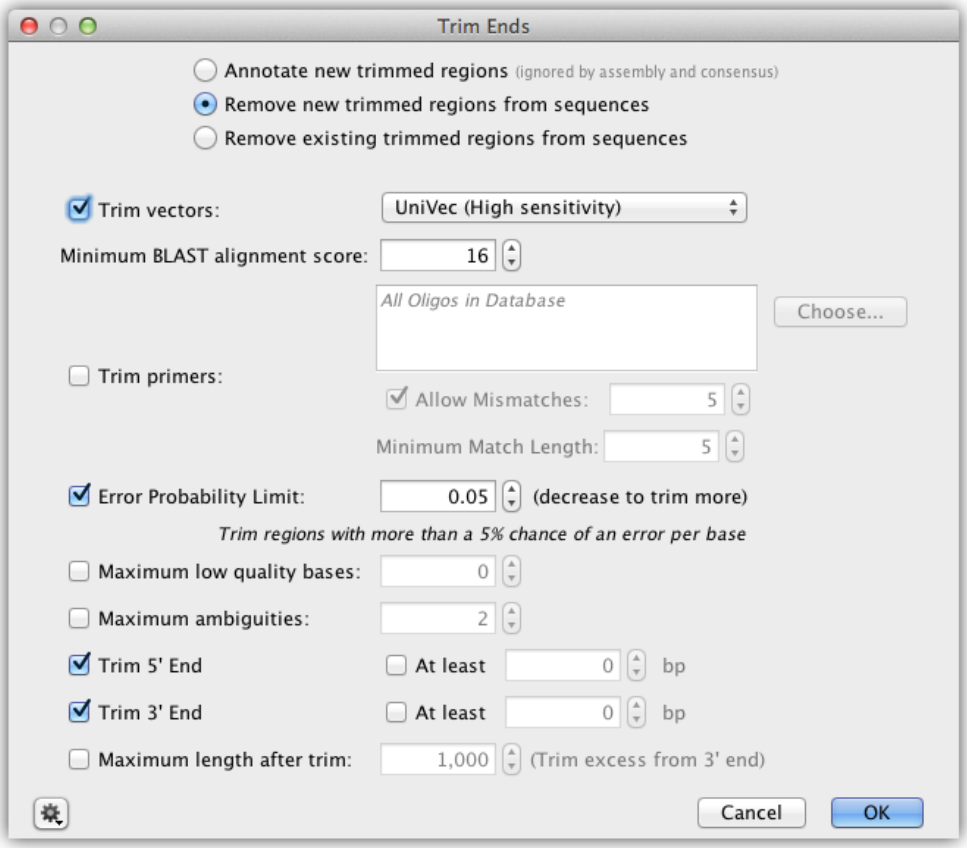


Figure 4.14: Trimming options

- **Annotate new trimmed regions:** Calculate new trimmed regions and annotate them - the trimmed regions will be ignored when performing assembly and calculating the consensus sequence.
- **Remove new trimmed regions from sequences:** Calculate new trimmed regions and remove them from the sequence(s) completely. This can be undone in the Sequence View before the sequences are saved.

- **Remove existing trimmed regions from sequences:** This is only available when there are already trimmed regions on some of the sequences. This will remove the existing trimmed regions from the sequences permanently; no new trimmed regions are calculated.
- **Trim vectors:** Screens the sequences against UniVec to locate any vector contamination and trim it. This uses an implementation similar to NCBI's VecScreen to detect contamination - (http://www.ncbi.nlm.nih.gov/projects/VecScreen/)
- **Trim primers:** Screens the sequences against primers in your local database.
- **Error Probability Limit:** Available for chromatogram documents which have quality (confidence) values. The ends are trimmed using the modified-Mott algorithm based on these quality values (Richard Mott personal communication). This trims bases up until the point where trimming further bases will only improve the error rate by less than the limit.
- **Maximum low quality bases:** Specifies the maximum number of low quality bases that can be in the untrimmed region. Low Quality is normally defined as confidence of 20 or less. This can be adjusted on the Sequencing and Assembly tab of Preferences.
- **Maximum Ambiguities:** Finds the longest region in the sequence with no more N's than the maximum ambiguous bases value and trims what is not in this region. This should be used when sequences have no quality information attached.
- **Trim 5′ End and Trim 3′ End:** These can be set to specify trimming of only the 3′ or 5′ end of the sequence. A minimum amount that must be trimmed from each end can also be specified.
- **Maximum length after trim:** If the untrimmed region is longer than the specified limit then the remainder will be trimmed from the 3′ end of the sequence until it is this length.

4.7.4 Using paired reads

To assemble paired read (or mate pair) data, prior to assembly you first need to tell Geneious the reads are paired and then the assembler will automatically used the paired data unless you turn off the advanced option to "Use paired distances". To set up paired reads, you need to select the document(s) containing the paired reads and select "Set Paired Reads" from the sequence menu. Depending on your data source, reads could be in parallel sets of sequences, or interlaced, so you need to tell Geneious which format. Geneious will guess and select the appropriate option based on the data you have selected, so most of the time you can just use the default value for this. However, you must make sure you select the correct "Relative Orientation" for your data. Different sequencing technologies orientate their paired reads differently. All paired read data will have a known expected distance between each pair. It is important you set this to the correct value to achieve good results when assembling. If you don't know

what the relative orientation or expected distance is between the reads you should ask your sequencing data provider.

When you click 'OK', of you chose to pair by parallel lists of sequences, Geneious will create a new document containing the paired reads. If you chose to pair an interlaced list of sequences (or modify settings for some already paired data), Geneious will just modify the existing list of sequences to mark it as paired.

If you choose to split reads based on the presence of a linker sequence (e.g. for 454 data) the original sequences will be unmodified and the split reads will be created in a new document. The default behaviour is to ignore sequences shorter than 4bp either side of the linker, but this can be customized from the "Edit Linkers" option in the paired reads options.

Polonator sequencing machine reads can be split using the "Split each read in half" option.

4.7.5 Splitting multiplex/barcode data

Multiplex or barcode data (e.g. 454 MID data) can be separated using "Separate Reads by Barcode" from the Sequence menu.

The barcode options allow for mismatches (substitutions, deletions or insertions) and trimming of primer fragments, adapter and linker sequences is also supported. All sequences matching a barcode are copied to an correspondingly named sequence list document.

Default settings are supplied for 454 MID data splitting so that it recognizes all 151 MID sequences provided by 454 and uses their names when appropriate. The 454 Adapter B sequence is trimmed from the end of the MID sequences.

For further information on splitting barcode data, hover the mouse over any of the settings in the "Separate Reads by Barcode" options window.

4.7.6 Viewing Contigs

Contigs in Geneious are viewed (and edited) in exactly the same way as alignments. There are several features in the sequence viewer which are worth taking special note of when viewing contigs:

- The consensus sequence is normally of particular interset and this is always displayed at the top of the sequence view (labeled Consensus).
- When all sequences in a contig (or alignment) have quality information attached then you can select the "Highest Quality" consensus type. This almost removes the need for manually editing the contig because this consensus chooses the base with the highest total quality at each position.

- There is a Quality color scheme which is selected by default for alignments of all chromatograms. This assigns a shade of blue to each base based on its quality. Dark blue for confidence < 20, blue for 20 - 40 and light blue for > 40. The consensus is also colored with this scheme where the confidence of a given base in the consensus is equal to the maximum confidence from the bases at that site in the alignment.
- The sequence logo graph has an option to "Weight by quality". This is very useful for identifying low quality regions and resolving conflicts.

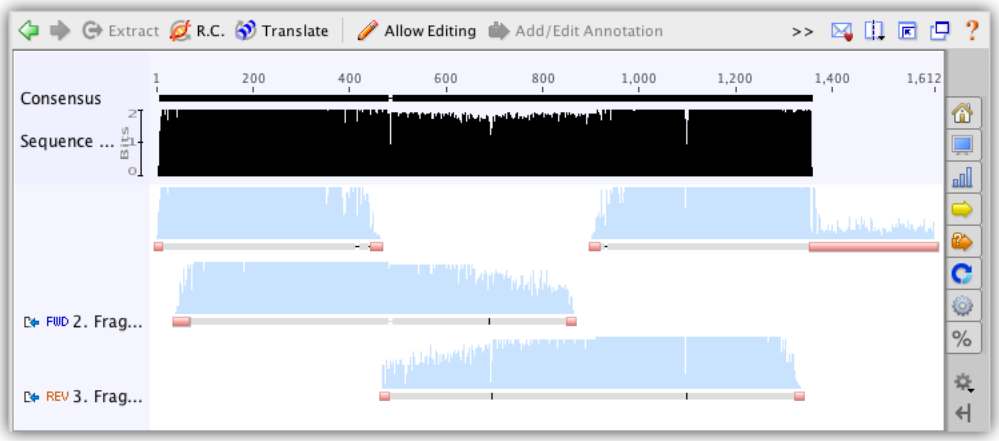


Figure 4.15: The overview of a contig

Finding disagreements or SNPs

To easily identify bases which do not match the consensus, turn on "Highlight Disagreements" in the consensus section of the sequence viewer options. When this is on, any base in the sequences which matches the consensus at that position is grayed out and bases not matching are left colored.

With this on you can quickly jump to each disagreement by pressing Ctrl+D (⌘+D on Mac OS X) or by clicking the "Next Disagreement" button in the sequence viewer option panel to the right. Each disagreement can then be examined or resolved.

You can also use this feature If you have aligned to a reference sequence and you are interested in finding differences between each sequence and the reference (or SNPs).

Manually investigating every little disagreement can be time consuming on larger contigs. There is also a "Find Variations/SNPs" feature from the "Annotate & Predict" toolbar which will annotate regions of disagreement and it can be configured to only find disagreements

above a minimum threshold to screen out disagreements due to read errors. This feature can also be configured to only find disagreements in coding regions (if the reference sequence has CDS annotations present) and can analyze the effects of variations on the protein translation to allow you to quickly identify silent or non-silent mutations. It can also calculate p-values for variations and filter only for variations with a specified maximum P-Value.

The p-value represents the probability of a sequencing error resulting in observing bases with at least the given sum of qualities. The lower the p-value, the more likely the variation at the given position represents an allele.

When calculating P-Values:

- The contig is assumed to have been fine tuned around indels
- Ambiguity characters are ignored (other characters in the column are still used)
- Homopolymer region qualities are reduced to be symmetrical across the homopolymer. For example if a series of 6 Gs have quality values 37, 31, 23, 15, 7, 2 then these are treated as though they are 2, 7, 15, 15, 7, 2. This is done because variations may be called at either end of the homopolymer and because reads may be from different strands.
- Gaps are assumed to have a quality equal to the minimum quality on either side of them (after adjusting for homopolymers)
- When finding variations relative to a reference sequence, the p-value calculated is for the variant, not the change. In other words the p-values calculated are independent of the reference sequence data.
- The approximate p-value method calculates the p-value by first averaging the qualities of each base equal to the proposed SNP and averaging the qualities of each base not equal to the proposed SNP.
- Example: Assume you have a column where the reference sequence is an A and there are 3 reads covering that position.

  1 read contains an A in the column and the other 2 reads contain a G. All 3 reads have quality 20 (= 99% confidence) at this position

  We want to calculate the p-value for calling a G SNP in this column. Since the quality values are all equal, the p-value is the probability of seeing at least 2 Gs if there isn't really a variant here, which is equal to ${}^3C_2 * 0.01^2 * 0.99 +^3 C_3 * 0.01^3$

False SNPs due to strand-bias (when sequencing errors tend to occur only on reads in a single direction) can be eliminated by specifying a value for the 'Minimum Strand-Bias P-value' setting. A 'Strand-Bias P-Value' property is added to each SNP to indicate the probability of seeing a strand bias at least this extreme assuming that there is no strand bias. SNPs with a smaller strand bias p-value will be excluded from the results when using this setting.

For full details of how the various settings work in the Variation/SNP finder, hover the mouse over them in Geneious to read the tooltips or click one of the '?' buttons.

The output of the Variant/SNP finder includes the following fields

- **Coverage**: The number of reads that cover the SNP region in the contig. The coverage includes both the reads containing the SNP and other reads at that position.
- **Reference Frequency**: The percentage of reads that agree with the reference sequence at that position. This field will only be present if at least 1 read agrees with the reference sequence.
- **Variant Frequency**: The percentage of reads that have the variation at that position. For variations that span more than a single nucleotide, the variant frequency may appear as a range (e.g. 47.8% – 51.7%) to indicate the minimum/maximum variant frequency over that range.
- **Polymorhpism Type**: This may be one of the following.

  SNP (Transition): a single nucleotide transition change from the reference sequence

  SNP (Transversion): a single nucleotide transversion change from the reference sequence

  SNP: At a single position, there are multiple variations from the reference sequence

  Substitution: A change of 2 or more adjacent nucleotides from the reference sequence

  Insertion: 1 or more nucleotides inserted relative to the reference sequence

  Deletion: 1 or more nucleotides deleted relative to the reference sequence

  Mixture: multiple variations from the reference sequence which are not all the same length
- **Change**: Indicates the reference sequence nucleotides followed by the variant nucleotides. For example 'C → A'

For variations inside coding regions (CDS annotations) the following fields may be present

- **Codon Change**: indicates the change in codon. Essentially this is the same as the 'Change' field, but extended to include the full codon(s). For example 'TTC → TTA'
- **Amino Acid Change**: indicates the change (if any) in the amino acid(s) by translating the codon change. For example 'F → L'
- **Protein Effect**: summarizes the change on the protein as either a substitution, frame shift, truncation (stop codon introduced) or extension (stop codon lost)

Finding regions of low/high coverage

In addition to the coverage graph which gives you a quick overview of coverage, under then "Annotate & Predict" toolbar is the "Find Low/High Coverage" feature. This feature annotates all regions of low/high coverage which you can then navigate through using the little left and right arrows next to the coverage annotations in the controls on the right. You can set the threshold low/high coverage by either specifying an absolute number of sequences or a number of standard deviations from the mean coverage.

Viewing Contigs of Paired Reads

In order to view a contig of paired reads, you first need to have set up the paired data before assembling - see 4.7.4. Once you have your paired read assembly, the contig viewer adds an option to "Link paired reads" in the advanced section of the controls on the right. This means that pairs of reads will be laid out in the same row with a horizontal line connecting them. Reads separated by more than 3 times their expected distance are not linked by default unless the "Link distant reads" setting is turned on.

The horizontal line between paired reads is colored according to how close the separation between the reads is to their expected separation. Green indicates they are correct, orange and blue indicate under or over their expected separation and red indicates the reads are incorrectly orientated.

The reads themselves can also be configured to be colored in this way if you use the "Paired Distance" color scheme from the general (top section in the controls on the right) settings. The colors used and the sensitivity for deciding if reads are close enough to their expected distance can be configured from the 'Options' link when the 'Paired Distance' color scheme is selected.

You can hover the mouse of any read in a contig and the status bar will indicate the expect separation and expected separation between the reads.

4.7.7 Editing Contigs

Editing a contig is exactly the same as editing an alignment in Geneious. After selecting the contig, click the 'Edit' button in the sequence viewer and you can modify, insert and delete characters like in a standard text editor.

Editing of contigs is done to resolve conflicts between fragments before saving the final consensus. The normal procedure for this is to look through the disagreements in the contig (as described above) and change bases which you believe are bad calls to be the base which you believe is the correct call. This is often decided by looking at the quality for each of the bases and choosing the higher quality one. Geneious can do this automatically for you if you use the "Highest Quality" consensus.

Bases in the consensus sequence can also be edited which will update every sequence at the corresponding position to match what is set in the consensus.

Figure 4.16: Highlight disagreements and edit to resolve them

4.7.8 Saving the Consensus

Once you are satisfied with a contig you can save the consensus as a new sequence by clicking on the name of the consensus sequence in your contig and clicking the 'Extract' button.

4.8 Saving operation settings (option profiles)

Profiles allow you to save the settings for almost any analysis operation in Geneious so they can be loaded later or shared with others. Eg. the recommended trimming parameters for your organization can be saved as a profile and then shared on the shared Database for everyone to use.

This button appears in the bottom-left corner of any options window where profiles can be saved and loaded. Click on this button to reset to defaults, load a profile, save a new profile or manage your existing profiles.

4.8.1 Saving a profile

To create a profile, set the options up the way you want then choose 'Save Current Settings'. You can then enter a name for your profile and choose whether it is shared. For a description of shared profiles see the section on sharing profiles.

When you save a profile it is attached to the particular analysis window that you have open. Eg. if you save a profile for Alignment it can only be loaded for Alignment, not for Assembly.

4.8.2 Loading a profile

To load a profile, choose 'Load Profile' and click on the name of the profile you want to load. The settings for the operation will immediately update to reflect the profile.

Note: Sometimes when you load a profile the settings may not exactly match what was saved. This is because the available settings can change depending on what type of documents you have selected.

4.8.3 Managing profiles

Click on 'Manage Profiles' under 'Load Profile' to see a list of profiles with options for deleting, editing, importing and exporting profiles. See sharing profiles section below for more on import and export.

4.8.4 Sharing profiles

There are two ways to share option profiles:

- Import and export from the 'Manage Profiles' window allows you to save a file containing a particular profile. These can be emailed to other Geneious users and imported for use with their data. The easiest way to import a profile is by dragging the file directly in to Geneious.
- If a profile is marked as 'Shared' (when it was created or by editing it) then the profile will be copied across to any Shared Database that you connect to. This means anyone else who connects to the same Shared Database will automatically have the profile under their 'Load Profile' menu. *Note*: Once a profile is shared it cannot be un-shared, but it can be deleted. Also, other users can edit or delete a shared profile at any time.

4.9 Results of analysis

All analysis results are deposited in the currently selected folder. If no local folder is selected then you will be prompted for a local folder. This applies to sequence alignments, phylogenetic trees, sequence translations, reverse complements and extraction of sequences. Once generated, analysis results can be dragged to another location if desired.

Chapter 5

Custom BLAST

Custom BLAST allows you to create your own custom database from either FASTA files or sequences in your local folders, and BLAST against it.

5.1 Setting Up

The Custom BLAST plugin requires access to NCBI BLAST (not BLAST+) binary files.

5.1.1 Setting up the Custom BLAST files yourself

If you want, you can download or otherwise acquire the NCBI BLAST binary files outside of Geneious. You can download them from here:

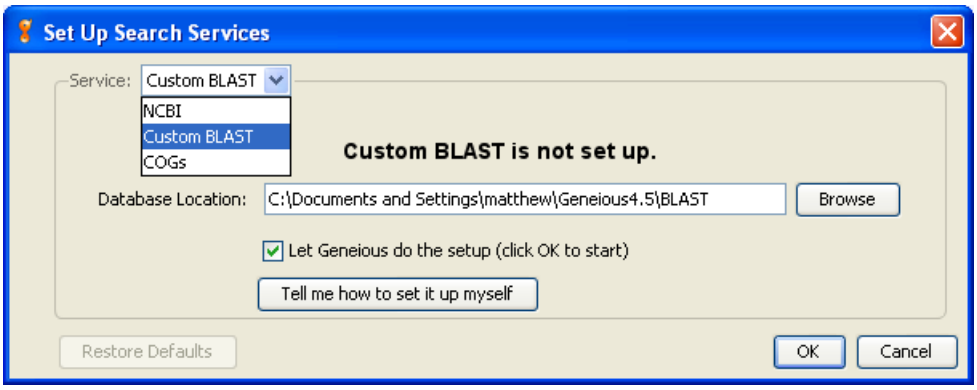
ftp://ftp.ncbi.nih.gov/blast/executables/release/LATEST

Choose the appropriate file for your operating system, download and extract it. You will need to let Geneious know where to look for the files once you have done this. To do this, go to "Tools"→"Add/Remove Databases"→"Set Up Search Services" and select "Custom BLAST" from the Service drop-down box. Enter your data location or click "Browse" to browse to the location of the files.

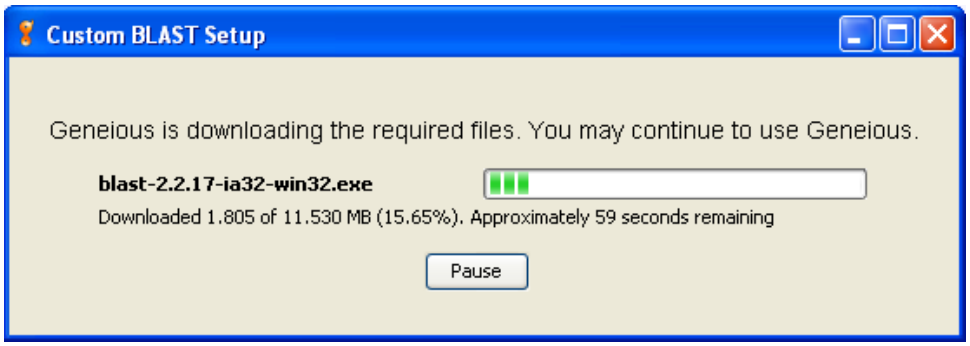
Note: If you decide to use executables for another version of BLAST, then make sure to use the legacy executables and not the newer BLAST+ executables that are not compatible with Geneious.

5.1.2 Setting up the Custom BLAST files through Geneious

Geneious provides a download manager to help you download and extract the Custom BLAST files. To use it, go to "Tools"→"Add/Remove Databases"→"Set Up Search Services" and select "Custom BLAST" from the Service drop-down box. Make sure "Let Geneious do the setup" is checked. Then click 'OK'. After a few seconds the compressed file containing all the files needed to run Custom BLAST will start downloading. You can click 'Pause' to pause the download. You can add and search Custom BLAST databases as soon as it has finished downloading and extracting. If you shut down Geneious with the file partially downloaded, you will need to start downloading it again from the beginning.



(a) Setup Options


Custom BLAST Setup
Geneious is downloading the required files. You may continue to use Geneious.
blast-2.2.17-ia32-win32.exe
Downloaded 1.805 of 11.530 MB (15.65%). Approximately 59 seconds remaining
Pause

(b) Downloading

Figure 5.1: Setting Up Custom BLAST

5.1.3 Adding Databases

Now that you have set up the executables, it is time to add databases to your BLAST.

Adding FASTA databases

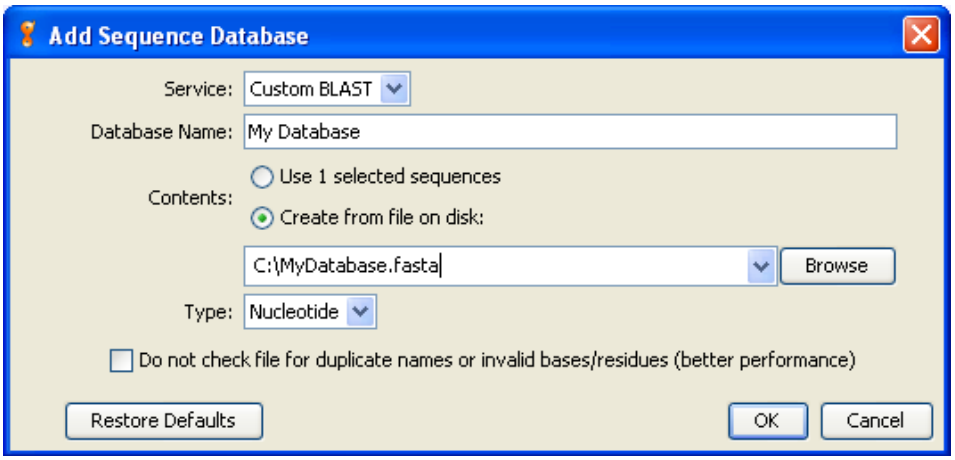


Figure 5.2: Adding a FASTA database

To create a database from the sequences in a FASTA file, go to "Tools"→"Add/Remove Databases"→"Add Sequence Database" and select "Custom BLAST" from the Service drop-down box. Choose to "Create from file on disk" and then click 'Browse' to navigate to the FASTA file that contains the sequences you want to BLAST. Enter a name for the database and click 'OK'. There are two requirements for a FASTA file to be suitable for creating a database from:

- The FASTA file must contain only the same types of sequence (i.e. Nucleotide or Amino Acid)
- The sequences in the FASTA file must all have unique names

If the file meets these requirements it will be added as a database, otherwise you will be informed of the problem.

Creating a database from local documents

To create a BLAST database from sequences in your local documents folders, first select the documents that you want. Then go to "Tools"→"Add/Remove Databases"→"Add Sequence Database" and select "Custom BLAST" from the Service drop-down box. Enter a name for the database, and click 'OK'.

5.1.4 Using Custom BLAST

Once you have added one or more databases, they will appear under Custom BLAST in the "Sequence Search" database drop down. These can be used in exactly the same way as the NCBI BLAST ones.

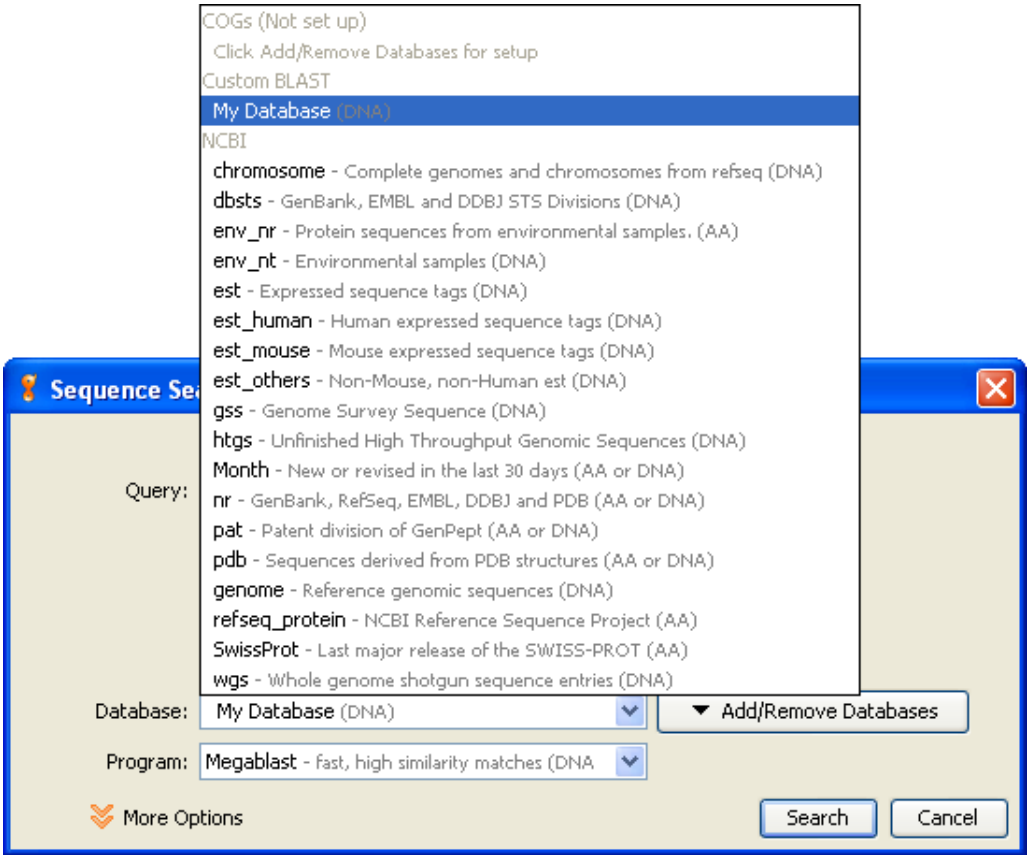

COGs (Not set up)
Click Add/Remove Databases for setup
Custom BLAST
My Database (DNA)
NCBI
chromosome - Complete genomes and chromosomes from refseq (DNA)
dbsts - GenBank, EMBL and DDBJ STS Divisions (DNA)
env_nr - Protein sequences from environmental samples. (AA)
env_nt - Environmental samples (DNA)
est - Expressed sequence tags (DNA)
est_human - Human expressed sequence tags (DNA)
est_mouse - Mouse expressed sequence tags (DNA)
est_others - Non-Mouse, non-Human est (DNA)
gss - Genome Survey Sequence (DNA)
htgs - Unfinished High Throughput Genomic Sequences (DNA)
Month - New or revised in the last 30 days (AA or DNA)
nr - GenBank, RefSeq, EMBL, DDBJ and PDB (AA or DNA)
pat - Patent division of GenPept (AA or DNA)
pdb - Sequences derived from PDB structures (AA or DNA)
genome - Reference genomic sequences (DNA)
refseq_protein - NCBI Reference Sequence Project (AA)
SwissProt - Last major release of the SWISS-PROT (AA)
wgs - Whole genome shotgun sequence entries (DNA)
Sequence Se
Query:
Database:
My Database (DNA)
Add/Remove Databases
Program:
Megablast - fast, high similarity matches (DNA
More Options
Search
Cancel

Figure 5.3: Searching a Custom BLAST database

Chapter 6

COGs BLAST)

COGs BLAST allows you to BLAST against the COGs database (http://www.ncbi.nlm.nih.gov/COG/). Geneious will BLAST your sequence against the COGs database, identify which COG the sequence is most likely to reside in, and give you information about the COG.

6.1 Setting Up

To set up the COGs database, you first need to set up Custom BLAST on your computer (see the section on Custom BLAST). Once you have set up Custom BLAST, you need to set up the COGs database files.

6.1.1 Downloading the COGs BLAST files yourself

If you want, you can download or otherwise acquire the COGs BLAST database files outside of Geneious. You can download them from here:

(ftp://ftp.ncbi.nih.gov/pub/COG/COG/).

The files you need are:

- myva
- myva=gb
- whog

Save these files to a local folder. Now go to "Tools"→"Add/Remove Databases"→"Add Sequence Database" and select "Custom BLAST" using the Service drop-down box. Choose to

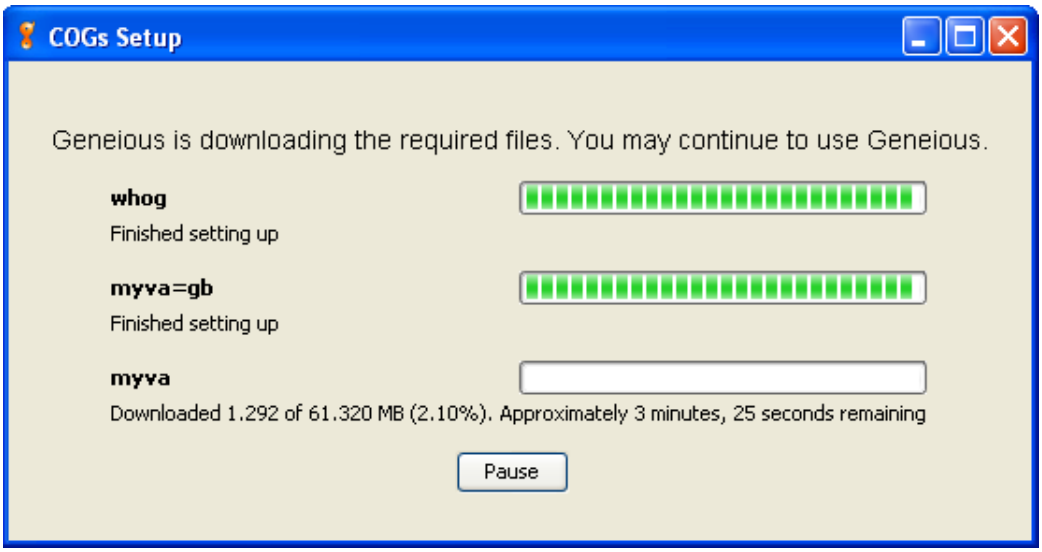

COGs Setup
Geneious is downloading the required files. You may continue to use Geneious.
whog
Finished setting up
myva=gb
Finished setting up
myva
Downloaded 1.292 of 61.320 MB (2.10%). Approximately 3 minutes, 25 seconds remaining
Pause

Figure 6.1: The Cogs BLAST Download Manager

"Create from file on disk" and click 'Browse'. Navigate to the file myva and click 'OK' (make sure that the protein database option is checked). Now copy the other two files that you downloaded into the data folder inside your Custom BLAST folder.

6.1.2 Downloading the COGs BLAST databases through Geneious

Geneious provides a download manager to help you download and set up the COGs BLAST database. To use it, go to "Tools"→"Add/Remove Databases"→"Set Up Search Services" and select "COGS BLAST" from the Service drop-down box. Make sure "Let Geneious do the setup" is checked. Then click 'OK'. After a few seconds the compressed file containing all the files needed to run COGS BLAST will start downloading

You can click 'Pause' to pause the download. Once all the files have finished downloading and setting up, you will need to close the dialog. If you shut down Geneious with a file partially downloaded, you will need to start downloading it again from the beginning. Files completely downloaded will not need to be downloaded again.

6.2 BLASTing COGs

Select any sequence in the document table, right click it, and select "Sequence Search". Select the COGS database from the database drop-down box and Geneious will give you several options for your blast (see Figure 6.2). Number of hits to fetch allows you to fetch results for

the best *n* hits for your sequence. You can choose to download COGs sequence from NCBI (with full annotations) or to load them without annotations from the COGs database file. Finally you have the option of retrieving the sequences for your hits, the entire COG for each hit, or to just display information about the hits. Once you have made your choices, click 'OK'. If you have selected a Nucleotide sequence, Geneious will give you options to translate it at this point.

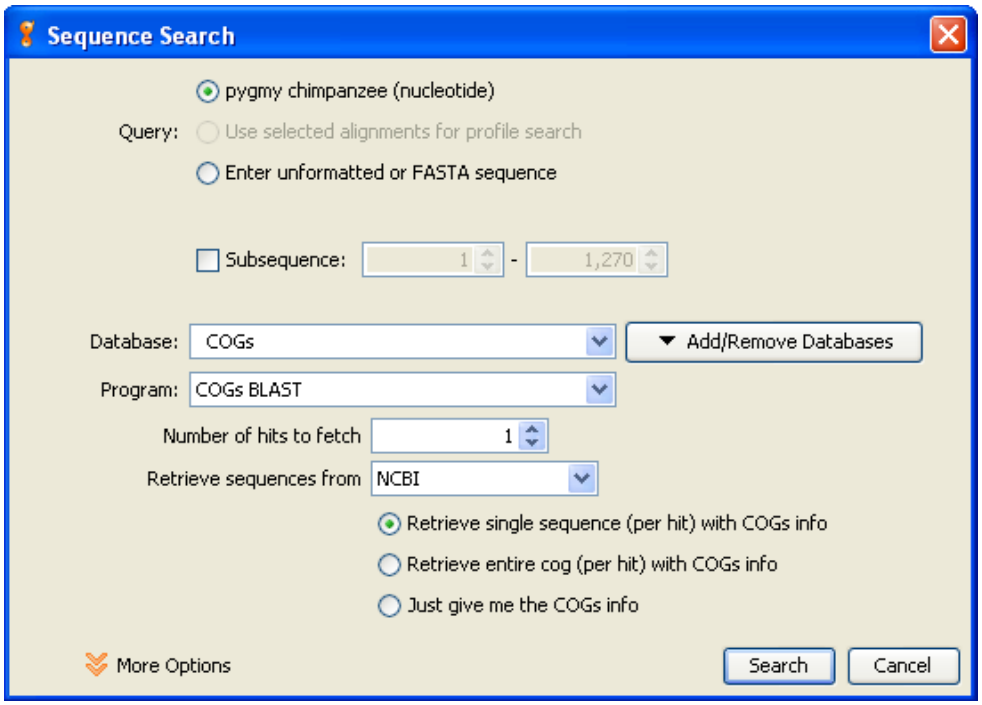


Figure 6.2: Configuring a COGs BLAST

Chapter 7

Pfam

Pfam is a large collection of multiple sequence alignments and hidden Markov models covering many common protein domains and families. The data for Pfam is taken from sequences in UniProt. Pfam can be found online at the following locations:

- Sanger Institute (UK)
- Washington D.C. (USA)
- Karolinska Institutet (Sweden)
- Institut National de la Recherche Agronomique (France)

7.1 Setting up the Pfam databases

At the time of release of Geneious 3.5, there was no public online interface to the Pfam database, (although there is one in the works at the Sanger Institute). For this reason, if you want to search the Pfam databases, you will need to download them first. As of Pfam 22 (July 2007) the subset of the Pfam databases used by Geneious totalled about 4GB in size, so it is recommended you download them somewhere with a fast connection.

You can use Geneious to search five of the Pfam databases:

1. **Pfam-A.seed** (29 MB) contains records on the manually curated domains in Pfam-A and the seed alignment (alignment of a representative subset of all occurrences of this domain in UniProt sequences) for each domain
2. **Pfam-A.full** (392 MB) contains records for the manually curated domains in Pfam-A and the full alignment (alignment of all occurrences of this domain in UniProt sequences) for each domain

3. **Pfam-B** (59 MB) contains records for the automatically generated domains in Pfam-B taken from PRODOM
4. **Pfam-C** (69 KB) contains records for Pfam clans (families of similar domains)
5. **swisspfam** (132 MB) contains data on the domain architecture of UniProt sequences.

7.1.1 Downloading the Pfam databases yourself

If you want, you can download or otherwise acquire the Pfam databases outside of Geneious. You will need to let Geneious know where to look for the files once you have done this. To do this, select the Pfam service. Click the "Change Database Location" and browse to the location of the databases.

7.1.2 Downloading the Pfam databases through Geneious

Geneious provides a download manager to help you download the Pfam files. To use it, select the Pfam Service. Click the "Let Geneious do it" button. Then click the 'Start' button. After a few seconds the first database will start downloading. You can click 'Pause' to pause the download. You can search a database as soon as it has finished downloading and its contents have been verified. If you shut down Geneious with a file partially downloaded, you will need to start downloading it again from the beginning.

The Pfam databases total around 4 GB in size, most of which comes from Pfam-A.full. If your internet connection is slow or you have a low data cap you may want to download the databases elsewhere, and then transfer them to your computer. You may also consider downloading all databases except Pfam-A.full.

7.2 Pfam Document Types

There are three special document types used for Pfam data:

Pfam sequence documents are based on UniProt sequences. They contain all the information from the UniProt sequence, plus information on the Pfam domains in the sequence. You can view the domains as annotations in the sequence view, or on their own from the domain view.

Domain documents contain information about Pfam A full, Pfam A seed and Pfam B domains. This includes general information about the domain, references (visible in the reference view) and the alignment for the domain.

Clan documents contain information about a clan, including general information, refer-

ences (visible in the reference view) and a list of the domains which are members of this clan.

7.3 Pfam Operations

There are a number of special operations available to Pfam documents and UniProt sequences. To take advantage of these operations, you will need to have the Pfam databases set up.

The following Pfam operations are available:

- **Create Pfam Sequence** creates a Pfam sequence document from a UniProt sequence. You can view the domain information in a Pfam sequence document using the Domain Viewer. This operation can take a long time.
- With **Find Similar Sequences** you can search and create documents for sequences in UniProt which match the domain architecture of your Pfam sequence document, ie they have the same domains in the same places. This operation can take a long time.
- **Get Domains in Sequence** creates a domain document for every domain in a Pfam sequence document.
- If your domain document is a member of a Pfam clan, you can use **Get Clan** to get a document representing that clan.
- **Get Domains in Clan** will do the opposite, ie get documents representing each domain in a clan.
- If your domain document contains the seed alignment for the domain, you can use **Get Full Alignment** to get a domain document with the full alignment.
- Conversely, you can use **Get seed alignment** to get a domain document with the seed alignment only from a domain document with the full alignment.
- **Get Full Sequences** will return the full UniProt sequence documents from which the sequences in the alignment in a domain were extracted.
- **Get Full Sequence** will return the full UniProt sequence document from which a sequence taken from an alignment in a domain was extracted.

Chapter 8

Geneious Education

This feature allows a teacher to create interactive tutorials and exercises for their students. A tutorial consists of a number of HTML pages and Geneious documents. The student edits the pages and documents to answer the tutorial questions, and then exports the tutorial to submit for marking.

8.1 Creating a tutorial

The backbone of Geneious Tutorials are the HTML documents. Simply create your documents, and place them together in a folder. If you make a page called "index.html", it will be treated as the main page. Geneious will follow all hyperlinks between the pages, and external hyperlinks (beginning with `http://`) will be opened in the user's browser. If you want to include figures and diagrams in the pages, just put the image files in the folder and reference them with `<img>` tags like a normal HTML document *(supported image formats are GIF, JPG, and PNG)*.

If you want to include Geneious documents in your tutorial, simply place them in the folder as above and they will automatically be imported into Geneious with the tutorial. If you want to link to them from the tutorial pages, create a hyperlink pointing to the file in the HTML document. For example, to create a link to the file sequence.fasta in your tutorial folder, use the HTML `<a href="sequence.fasta">click here</a>`. To open more than one document from a link, separate the filenames with the pipe *(|)* character, for example `<a href="sequence.fasta|sequence2.fasta">click here</a>`. Note that geneious files must contain only one document to be imported automatically with the tutorial.

You can add a short one-line summary by writing your summary in a file called *"summary.txt"* (case sensitive) and putting it in the tutorial folder. Make sure that the entire summary is on the first line of the file, as all other lines will be ignored.

Once you have all your files together, put the contents of the folder in a zip file with the exten-

sion *.tutorial.zip*. Be careful not to put subfolders in your zip file, as these are not supported.

8.2 Answering a tutorial

Import the tutorial document into Geneious (use "File" → "Import" → "From file"). The tutorial document and any associated geneious documents will be imported into the currently selected folder. The tutorial itself will be displayed in the help pane on the right hand side of the Geneious window. If you accidentaly close the help pane, you can display it by choosing 'Help' from the "Help" menu.

If the tutorial requires you to enter answers, click the edit button at the top of the tutorial window and type your answer in to the space provided. Click the 'Save' button when you are done.

If the tutorial has a link to a Geneious document, when you click the link the document will be opened in the document viewer. Any changes you make to this document will be preserved when you export the tutorial.

When you have finished the tutorial, export it by selecting the tutorial document and choosing "File" → "Export" →"Selected Docuemnts" from the main menu. Make sure that "Geneious Tutorial File" is selected as the filetype, and then give it a name and click 'Export'.

Chapter 9

Collaboration

Collaboration is an external plugin.

Collaboration allows Geneious users to share the products of their research and work with each other. Based on an open Internet protocol called *XMPP* or *Jabber*, it allows you to maintain a list of contacts, so that you see who is online when you sign on yourself. You can then share documents with your online contacts, and browse and work with their documents in return. The list of contacts is stored on the server, so you can easily access an account including its contacts both at work and on your private computer.

Collaboration can work with any existing Jabber service, such as Google Talk, but we recommend using the Geneious default, `talk.geneious.com`.

You can even access several Jabber accounts at the same time, which is particularly convenient if you wish to set up and run your own Jabber server (section 9.5.3).

This chapter shows you how to:

- Create a new collaboration account
- Search for, and add contacts to your account
- Share local folders with your contacts
- Search your contacts as you would an online database
- Set up and run your own Jabber server

9.1 Managing Your Accounts

When you start Geneious you will see the empty Collaboration service in the Services Panel and the "Collaboration" submenu under "Tools". You can open the "Add New Account" dialog by either right-clicking (Ctrl+click on Mac OS X) on Collaboration in the Services Panel and clicking, "Add New Account" in the popup menu, or by selecting the same option from the "Collaboration" submenu.

9.1.1 Add New Account

In this dialog you are given the options of creating a new account on the server or entering the details for an existing account, e.g. if you want to access an account from an additional computer. If you choose to create a new account Geneious will attempt to automatically register your account on the server at the end of this process.

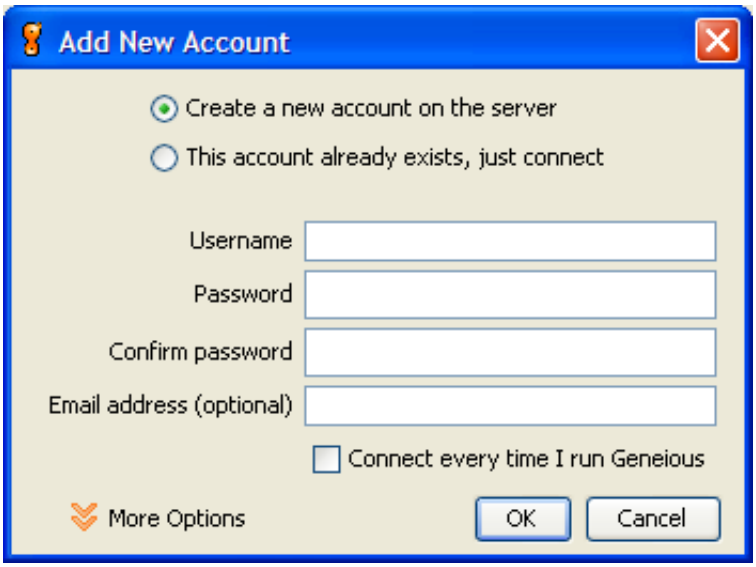


Figure 9.1: Add New Account dialog box

Choose a username and password now. Enter your password twice for a new account.

You can also optionally add an email address. Biomatters will need this if you require support regarding, e.g. reset of password or deletion of accounts.

More Options You can change some of the defaults for new and exiting accounts:

- *Account Name* is the name displayed in the Services Panel for this account. It defaults to your username if nothing is entered

- *Server* is the server your account connects to (default: `talk.geneious.com`).
- *Jabber Service Name* is required by some other Jabber service providers, such as Google Talk. Don't enter anything here unless you know what you are doing.
- *Port Number* for Jabber servers running on a non-standard port (default: 5222).

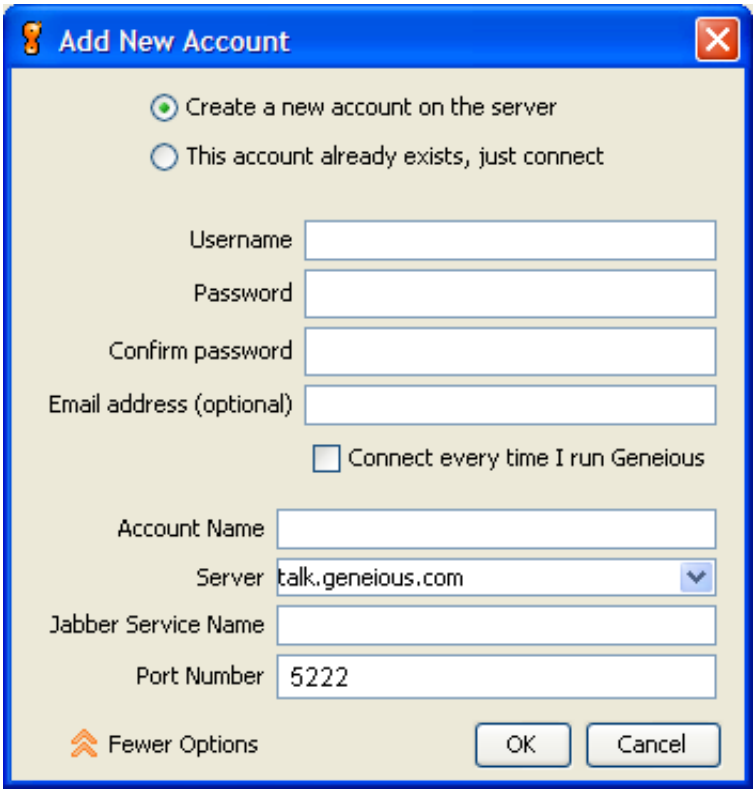


Figure 9.2: Add New Account dialog box with More Options

9.1.2 Edit Account Details

This option (from the "Collaboration" submenu, or your account's context menu) allows you to change the configuration you made when creating the account. If you change your password, Geneious will attempt to change it on the server the next time you connect. For this purpose, Geneious internally remembers your previous password as well, so that it can still connect if you have entered your new password while disconnected.

9.1.3 Connect/Disconnect

As all other collaboration-related commands, options for connecting to or disconnecting from your account are available both in the "Collaboration" submenu and your account's context meu (right-click, or on Ctrl+click on Mac OS X, on your account).

9.1.4 Delete Account

This option deletes your account configuration from Geneious. Currently, there is no option for deleting an account on the server.

9.2 Managing Your Contacts

Once you have an account and are connected you can start adding contacts. You will not be able to add contacts while an account is disconnected. Also, you will not be able to see a contact's online status until that contact has approved your request to do so.

9.2.1 Add Contact

Select your account in the Services Panel and choose "Add Contact" from the "Collaboration" submenu or right-click (Ctrl+click on Mac OS X) on your account in the Services Panel and choose the same option.

You will see a simple dialog with one field, Jabber ID. A Jabber ID looks like an email address and has a similar function: It uniquely identifies some other Geneious users account. You can enter a contact's Jabber ID directly into this field if you know it. To see your own Jabber ID hover your mouse over your account in the Services Panel and it will appear in a tool-tip.

Figure 9.3: Add Contact dialog box

If the server supports it, you should also see a "Search For Contact" link. Click this to go to the next dialog.

Here you will see a box for a search string, and some checkboxes indicating what you are searching on. Enter all or part of the name or email of the contact you want and click the 'Search' button. If any rows are returned in the results table you will be able to select one or entries and add them as contacts.

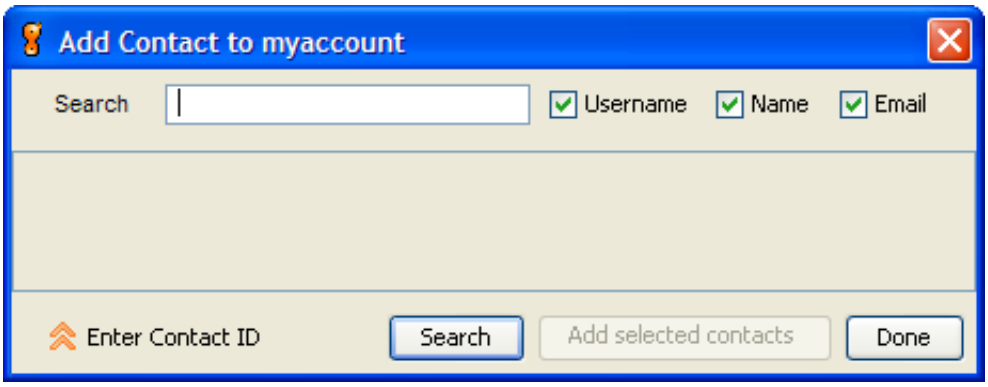


Figure 9.4: Add New Contact dialog box in searching mode

Your new contact will appear immediately in your contact list, however you will not be able to tell whether your new contact is online until they accept you as a contact. Similarly you will occasionally see a dialog box pop up asking you, "Allow user.name@talk.geneious.com as contact?" This is another Geneious user attempting to add you as a contact in this manner.

Your contact will appear grey in your contact list when they are offline. If your contact is online, they will appear blue. A contact online in Geneious will have the orange Geneious 'G' behind them. A contact online in some other program, like a chat client, whill have a speech bubble behind them.

9.2.2 Rename Contact

This option allows you to change the name that you know another contact by. This is the name the contact will appear under in the contact list and in chats; it is only visible to you.

9.2.3 Remove Contact

If you no longer wish to share documents with a contact, you can remove that contact by right-clicking (Ctrl+click on Mac OS X) the contact in the Services panel and selecting "Remove Contact...". This deletes you from their contact list as well. If you find that a contact has disappeared from your list, this may be the reason.

Figure 9.5: Rename Contact dialog box

9.3 Sharing Documents

Select one of your local folders. Select "Share Folder" from the "File" menu. Alternatively right-click (Ctrl+click on Mac OS X) on a local folder and select the same option.

- If you share a folder all documents in that folder are shared.
- If you share a folder all sub-folders of that folder are shared.
- If you share a folder it is available to all your contacts. In the future, Geneious may support per-account options for sharing your documents, or even organize contacts into groups so that you can share your documents with specific groups only.

9.4 Browsing, Searching and Viewing Shared Documents

Folders that your contacts have shared will appear beneath that contact just as they do in your contact's own Services panel. You can browse these folders as you do your local folders. You can also search a shared folder just as you can a local one.

Additionally, you can search all of a contact's shared documents by clicking on the contact itself and then conducting the search. You can also search all the shared documents of all of an account's contacts by clicking on the account and conducting the search. Agents can be set up on shared folders, contacts and accounts.

You cannot search, browse or run or set up agents on a contact that is currently offline.

When you first view your contact's documents in the Document Table, the documents you see are only summaries. To view the whole document, select the summary(s) of the documents(s) you would like to view and the click the "Download" button inside the document view or just above it. There are also "Download" items in the File menu and in the popup menu when document summary is right-clicked (Ctrl+click on Mac OS X). The size of these files is not

displayed in the Documents Table. You can cancel the download of document summaries by selecting "Cancel Downloads" from any of the locations mentioned above.

9.5 Chat

You can either chat with a single contact, or invite several contacts to join you in a new chat.

9.5.1 Chatting with One Contact

To start chatting with a particular contact (who may be online using Geneious or another chat client which uses the Jabber protocol), click on that contact and select "New Chat Session..." either from the "Collaboration" submenu or from the popup menu (right-click on the contact, or Ctrl+click on Mac OS X). Type your messages into the text field at the bottom of the window that pops up, and click 'Send' or press the 'Enter' key to send.

9.5.2 Chatting with Multiple Contacts

Starting a Chat Session with Multiple Contacts

To invite several contacts to join you in a new chat session, click on your account (not the contacts) and then select "New Chat Session..." from either the "Collaboration" submenu or the context menu (right-click on the account, or Ctrl+click on Mac OS X). Select the online contacts which you want to invite (you can select a range by Shift+clicking, or add contacts to the selection by Ctrl+clicking). Click 'invite' to create this new chat session.

Accepting or Declining an Invitation to Chat

When one of your contacts invites you to chat, a dialog will appear, asking you to accept or decline the chat invitation. Clicking 'Accept' will open a chat window that will allow you to chat with the contact who invited you, and with all other contacts that were invited. If you decline that invitation and enter a reason (optional), this reason will be displayed to everyone in the chat.

Sending and Viewing Messages in the Chat

The chat window displays your own and your contacts' previous messages. You can enter new messages in the field at the bottom. These messages will only be sent and become visible to your contacts once you click 'Send' or press the 'Enter' key.

To leave the chat, simply close the Chat Window.

9.5.3 Setting up and running your own Jabber server

Setting up your own Jabber server is simple and means that your documents will never leave your local network. This means that you will not have any problems with firewalls, achieve much greater download speeds, and it provides an extra security layer for the confidentiality of your documents, in case it is not sufficient for you that the communication with our Jabber server is encrypted, and that we do not log or share your data.

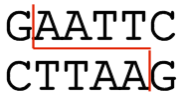
If you wish to set up and run your own Jabber server, we recommend using Openfire from Ignite Realtime [http://www.igniterealtime.org/projects/openfire/index.jsp/] which is available for free under the Apache 2.0 Open Source License [http://www.apache.org/licenses/LICENSE-2.0.html] Install and start the server on one computer, and then enter that computer's name or address in the "Server" field under "More Options", when creating a new account.

Please note that Biomatters cannot provide any further support for setting up and managing your Jabber server, except possibly under a contracting agreement.

Chapter 10

Cloning

Restriction Enzymes cut a nucleotide sequence at specific positions relative to the occurrences of the enzyme's *recognition sequence* in the sequence. For example, the enzyme *Eco*RI has the recognition sequence GAATTC and cuts both the strand and the antistrand sequence after the G inside the recognition sequence[1], leaving a single-stranded overhang (*sticky end (overhang)*):



The cloning features in Geneious allow you to identify candidate Restriction Enzymes[2] for your experiments and to determine *in silico* where they would cut your nucleotide sequences and which fragments they would produce. It also lets you ligate fragments and insert a fragment into a vector. If you select a nucleotide sequence, restriction analysis is available under the menu item Tools / Restriction Analysis, and in the context menu (right-click on the sequence, or Ctrl+click on Mac OS X):

- *Find Restriction Sites...* allows you to specify an arbitrary candidate set of restriction enzymes and the desired number of matches (so that you can e.g. identify enzymes that cut only once or twice), as well as a region enzymes may not cut within. After running the analysis, the position of the matching enzymes' recognition sequence and the sites where they cut will be visible on the sequence as annotations, and you will be able to see a table of all fragment start and end positions and their lengths, and of all restriction enzymes involved. These tables can be exported as .csv files for subsequent processing with other software such as e.g. Microsoft Excel®.

[1] Like many restriction enzymes *Eco*RI is methylation dependent and cuts only if the second A in the recognition sequence is not methylated to N6-methyladenosine.

[2] The restriction enzyme information included in Geneious was obtained from **Rebase** [18], available for free at http://rebase.neb.com.

- *Digest into fragments...* allows you to generate the actual fragments that would be created in a digestion experiment using restriction enzymes.. When running a digestion experiment, you can choose to either use the restriction sites already annotated to the sequences (or a subset that corresponds to only some specific enzymes), or you can let Geneious determine the cut sites for any candidate enzymes. The latter option finds the cut sites for the candidate enzymes and generates the fragments in a single step.
- *Ligate Sequences...* lets you ligate two or more fragments, with or without overhangs
- *Insert into Vector...* allows you to choose a digested fragment or a sequence with two restriction site annotations to use as an insert, and insert them into a vector (circular sequence). Geneious can do the work of working out what cut sites on the vector are compatible with the overhangs on the insert, with some additional information from you.
- *Gibson Assembly...* provides a one-step interface to perform a Gibson Assembly or similar operation (a isothermal ligase independent, restriction free overlap extension PCR cloning). You can chose to insert one or multiple inserts into one or multiple vectors and specify the insertion order. The operation automatically creates the necessary primers and the products you will get and generates a report document.
- *TOPO cloning...* automatically detects TOPO vectors amongst the selected sequences and inserts the fragments into these vectors using a BLUNT- TA- or Directional Cloning approach.

The following sections explain the more complicated operations in a little more detail.

10.1 Find Restriction Sites

The option *Find Restriction Sites...* from the "Tools"→"Cloning" menu or the context menu allows you to find and annotate restriction sites on a nucleotide sequence. You can configure the following options (Figure 10.1):

- *Candidate Enzymes* lets you select a set of restriction enzymes from which you want to draw the ones to use in the analysis. This will always include the option to use all known commercially available restriction enzymes, but if your search index is intact then all restriction enzyme set documents from your local database will also be listed (see below for how to create such a document).
- *Minimum effective recognition sequence length* lets you filter the candidate enzymes to include only ones whose recognition sequence has a given minimum effective length. For example, *Eco*RI's recognition sequence is 6 nucleotides long (GAATTC). The *effective* length takes ambiguities into account, so that e.g. the sequence YS only has an effective length of 1; it is a better measure for the expected number of hits in a random sequence of fixed

length, because YS matches CC, CG, TC and TG: On a random sequence with uniform nucleotide distribution it would match approximately once every nucleotide, as would a recognition sequence of length 1; hence, the *effective* length of YS is 1.

- *Only include enzymes that match X to Y times* lets you filter the results once the restriction sites have been identified. If checked, this option will discard all restriction sites for enzymes whose recognition sequence matches less than X or more than Y times. If you set X to be 0, when this operation is complete, it will report which candidate enzymes matched 0 times.
- *Exclude enzymes cutting between residues* lets you annotate only enzymes which do not cut within a certain range.
- If you select to show *More Options*, a table of all enzymes in your candidate set (filtered by the effective recognition sequence length constrained, when active) will be displayed. Only the enzymes selected in this table will be considered in the analysis; initially, all rows are selected. You can click on the column headers to sort the table ascending or descending by that column, and you can Shift+click and Ctrl+click to select a range of rows and to toggle the selection of a row, respectively.
- If not all candidate enzymes are currently selected (because of a recognition sequence length constraint, or because you have selected a subset of the table rows yourself), you can save the currently selected enzymes into a separate document by clicking *Save Selected Enzymes*. The document will be created in the current folder in your local database, and this set will then beavailable in the *Candidate Enzymes* option in this and all future analyses until the document is deleted. You can choose a custom name for the document, such as *Lab Fridge* or *Enzymes in pBlueScript II SK(+) multiple cloning site*.

After configuring your options, click 'OK' to start the analysis and annotate the restriction sites on the sequence, or 'Cancel' to abort.

10.2 Digest into fragments

The option *Digest into fragments...* from the Tools / Cloning menu or the context menu allows you to generate the nucleotide sequences that would result from a digestion experiment. You can digest multiple nucleotide sequences at a time. If the digestion results in overhangs, these will be recorded as annotations on the fragments.

- If you have selected only one nucleotide sequence document and it has annotated restriction sites, you can select *Digest using Annotated cut positions* to cut the document on these sites. When this option is selected, the options to filter the enzymes by their effective recognition sequence length or number of hits are disabled. However, if you select a subset of the enzymes under *More Options*, only the cut sites from these enzymes will

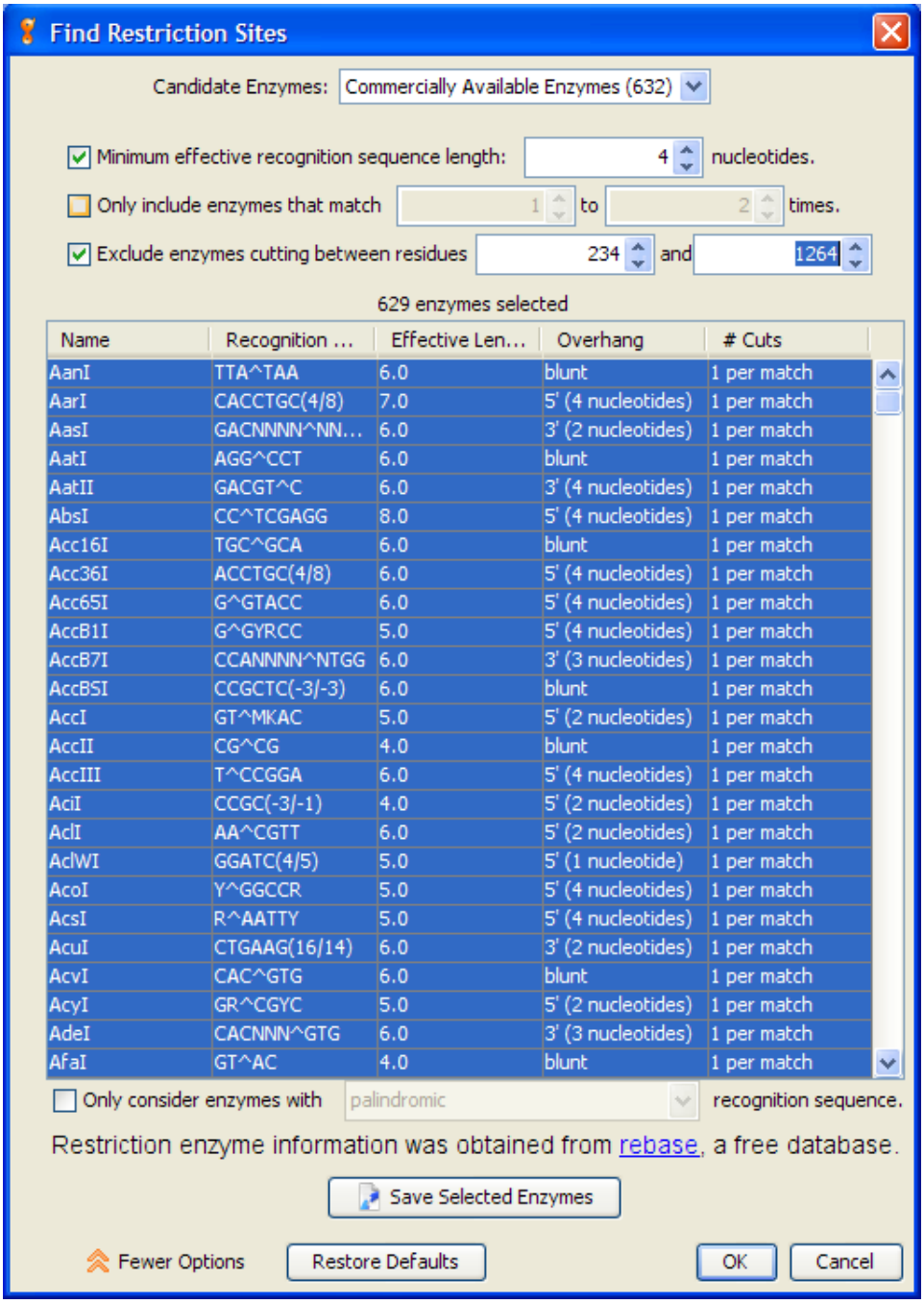

Find Restriction Sites
Candidate Enzymes: Commercially Available Enzymes (632)
Minimum effective recognition sequence length: 4 nucleotides.
Only include enzymes that match 1 to 2 times.
Exclude enzymes cutting between residues 234 and 1264
629 enzymes selected
Name | Recognition ... | Effective Len... | Overhang | # Cuts
AanI | TTA^TAA | 6.0 | blunt | 1 per match
AarI | CACCTGC(4/8) | 7.0 | 5' (4 nucleotides) | 1 per match
AasI | GACNNNN^NN... | 6.0 | 3' (2 nucleotides) | 1 per match
AatI | AGG^CCT | 6.0 | blunt | 1 per match
AatII | GACGT^C | 6.0 | 3' (4 nucleotides) | 1 per match
AbsI | CC^TCGAGG | 8.0 | 5' (4 nucleotides) | 1 per match
Acc16I | TGC^GCA | 6.0 | blunt | 1 per match
Acc36I | ACCTGC(4/8) | 6.0 | 5' (4 nucleotides) | 1 per match
Acc65I | G^GTACC | 6.0 | 5' (4 nucleotides) | 1 per match
AccB1I | G^GYRCC | 5.0 | 5' (4 nucleotides) | 1 per match
AccB7I | CCANNNN^NTGG | 6.0 | 3' (3 nucleotides) | 1 per match
AccBSI | CCGCTC(-3/-3) | 6.0 | blunt | 1 per match
AccI | GT^MKAC | 5.0 | 5' (2 nucleotides) | 1 per match
AccII | CG^CG | 4.0 | blunt | 1 per match
AccIII | T^CCGGA | 6.0 | 5' (4 nucleotides) | 1 per match
AciI | CCGC(-3/-1) | 4.0 | 5' (2 nucleotides) | 1 per match
AclI | AA^CGTT | 6.0 | 5' (2 nucleotides) | 1 per match
AclWI | GGATC(4/5) | 5.0 | 5' (1 nucleotide) | 1 per match
AcoI | Y^GGCCR | 5.0 | 5' (4 nucleotides) | 1 per match
AcsI | R^AATTY | 5.0 | 5' (4 nucleotides) | 1 per match
AcuI | CTGAAG(16/14) | 6.0 | 3' (2 nucleotides) | 1 per match
AcvI | CAC^GTG | 6.0 | blunt | 1 per match
AcyI | GR^CGYC | 5.0 | 5' (2 nucleotides) | 1 per match
AdeI | CACNNN^GTG | 6.0 | 3' (3 nucleotides) | 1 per match
AfaI | GT^AC | 4.0 | blunt | 1 per match
Only consider enzymes with palindromic recognition sequence.
Restriction enzyme information was obtained from rebase, a free database.
Save Selected Enzymes
Fewer Options
Restore Defaults
OK
Cancel

Figure 10.1: *Find Restriction Sites* options dialog, with extended options showing.

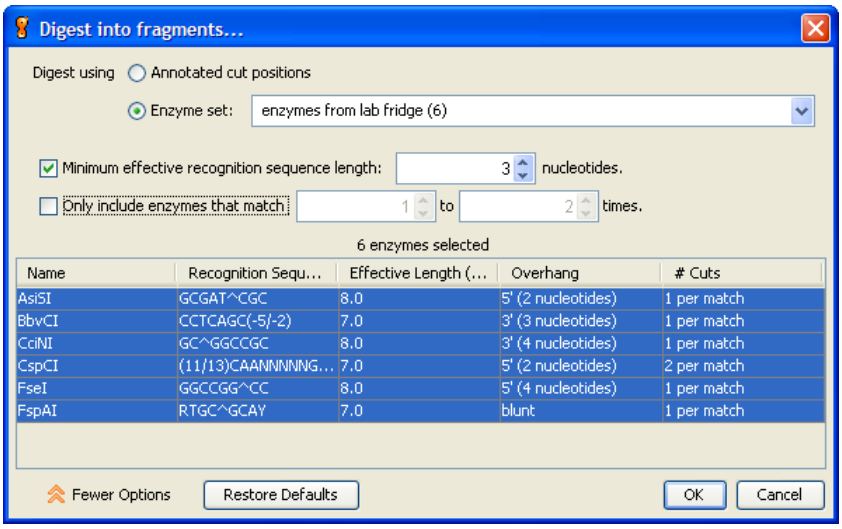


Figure 10.2: *Digest into fragments* options dialog, with extended options showing.

be considered; this can easily be used for the same effect by sorting by columns and then selecting a range of rows, in the rare cases when it is needed.

- Otherwise, if you select *Digest using Enzyme Set*, the digestion operation includes finding the restriction sites first (but without generating the annotations). Therefore, the options are the same as for *Find Restriction Sites...*, which is discussed in section 10.1.

10.3 Insert into Vector

The option *Insert into Vector...* from the Tools / Restriction Analysis menu or the context menu allows you to take an insert and insert it into a vector. The insert must be one of the following:

- A fragment which has already been digested. This fragment cannot have any restriction site annotations on it. The entire fragment will be inserted into the vector. Overhangs will be taken into account.
- A sequence with two restriction annotations. The fragment resulting from digesting this sequence (and discarding the fragments from the ends) will be inserted into the vector.

The vector must be a circular sequence. You do not need to annotate the restriction sites used to cut the vector in advance; the Insert into Vector operation will do that for you.

This operation cannot deal with some aspects of molecular cloning such as triple ligation and the blunting or filling in of overhangs. If you want to do a cloning operation outside the scope

of this operation, you will need to annotate restriction sites on the sequences involved, digest the fragments, modify them in the sequence viewer if necessary and then ligate them back together as a set of discrete steps.

10.3.1 Insert Options

You cannot alter the insert used in the operation from the options, but you can select what direction to insert in: forward or reverse. If the insert fragment has complementary overhangs or is blunt at both ends, you can also choose to insert in both directions. In this case, two product documents will be created, one for the insert in each direction.

The insert options also present a diagram showing the bases at each end of the insert fragment.

10.3.2 Vector Options

- *Polylinker (region to cut within):* These options let you choose what region within the vector sequence to look for enzymes to cut within. Geneious will examine the vector sequence for enzymes that have cut sites within this region and none outside it. You can specify the polylinker in the following ways:
  - *Annotation* If the vector has one or more polylinker annotations annotated on it, you can choose to use the interval covered by one such polylinker annotation directly.
  - *Bases* Used to explicitly specify the range of bases to use.
  - *Entire sequence* Used to specify that you can cut anywhere within the sequence.
- *Candidate Enzymes:* These options let you choose which enzymes to look for on the vector sequence
  - *Enzymes annotated on insert* This option lets you use only the enzymes used to cut the insert fragment.
  - *Enzyme set* This option lets you use the enzymes from a predefined enzyme set, eg. the enzyme set you have created containing the enzymes you have in your lab.
- *Cut vector with:* Whenever you change the options for the polylinker or candidate enzymes, Geneious will recalculate the compatible enzymes on the vector. It will look for enzymes which meet one of the following conditions (in addition to cutting only within the polylinker and belonging to enzymes from the candidate enzyme set):
  1. A single enzyme which cuts the vector once, such that the insert can be inserted in the gap (Possible only when the insert has complementary cut sites).
  2. A single enzyme which cuts the vector twice, such that the insert can be inserted into the gap vacated by the fragment between the two cut sites
  3. Two enzymes which each cut the vector once, such that the insert can be inserted into the gap vacated by the fragment between the two cut sites

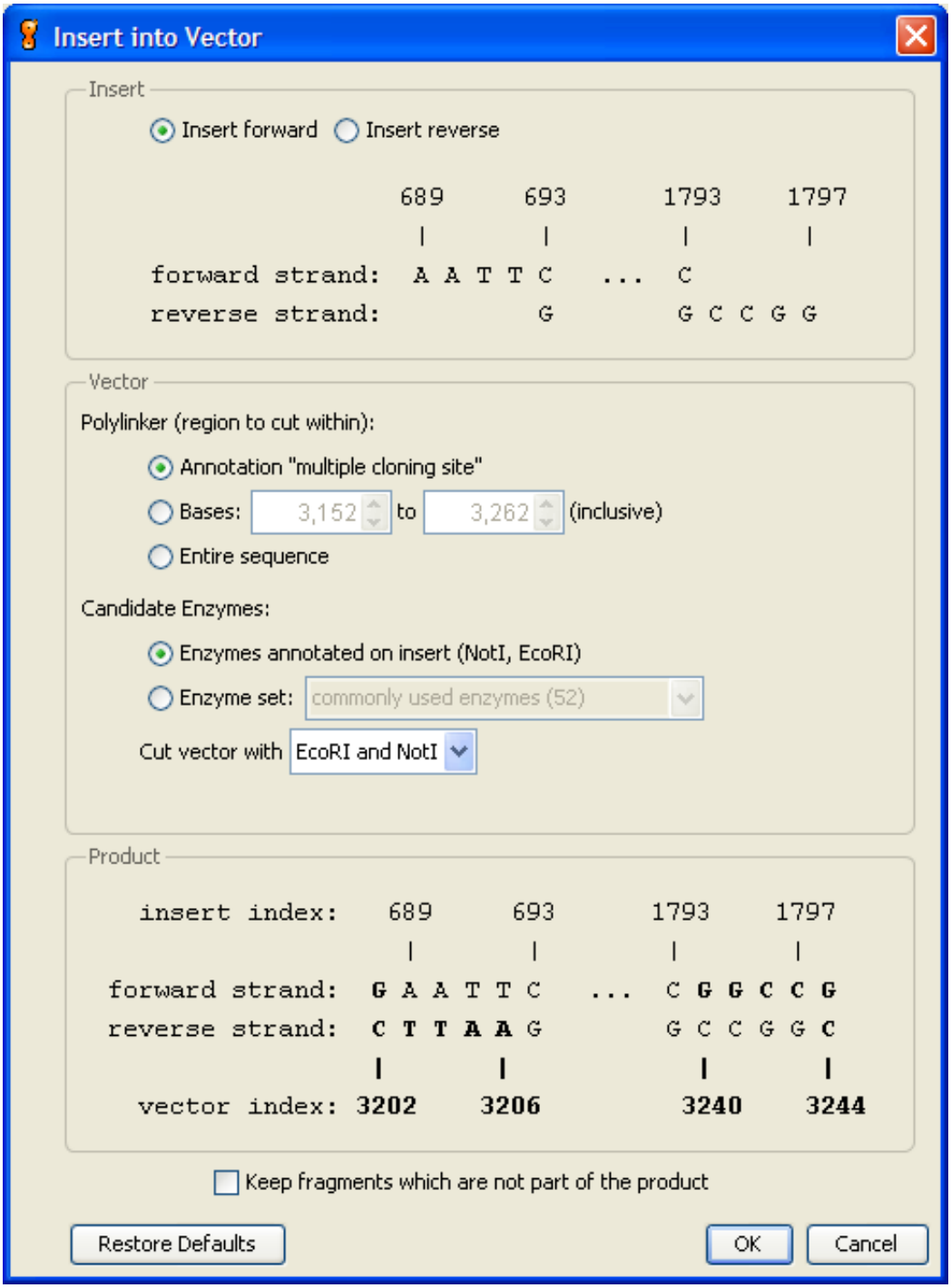

Insert into Vector
Insert
Insert forward
Insert reverse
689
693
1793
1797
forward strand: A A T T C ... C
reverse strand: G G C C G G
Vector
Polylinker (region to cut within):
Annotation "multiple cloning site"
Bases: 3,152 to 3,262 (inclusive)
Entire sequence
Candidate Enzymes:
Enzymes annotated on insert (NotI, EcoRI)
Enzyme set: commonly used enzymes (52)
Cut vector with EcoRI and NotI
Product
insert index: 689 693 1793 1797
forward strand: G A A T T C ... C G G C C G
reverse strand: C T T A A G G C C G G C
vector index: 3202 3206 3240 3244
Keep fragments which are not part of the product
Restore Defaults
OK
Cancel

Figure 10.3: *Insert into Vector* options dialog

10.3.3 Other Options

The Product section of the options displays a diagram showing the ligation points in the insertion. The parts of the ligation points belonging to the vector appear in bold in this diagram.

Below this is a checkbox where you can choose whether to *Keep fragments which are not part of the product*. If this box is checked, a document will be created representing the fragment removed from the vector, if any. If the insert fragment was produced from a sequence with two restriction site annotations, the fragments on either side of the restriction site annotations will also be kept.

10.4 Gateway® Cloning

Geneious contains three operations to assist with Gateway® cloning. Gateway is a registered trademark of Invitrogen Corporation.

10.4.1 Add AttB Sites

This operation allows you to add AttB sites to a PCR product. It will work on the following types of document:

- A PCR product. AttB sites will be appended to the PCR product.
- A document with primer binding sites annotated. If there is more than one pair, Geneious will ask you which pair to use. The PCR product will be extracted and AttB sites appended.

10.4.2 Gateway

This operation will perform a BP reaction or an LR reaction on the selected documents, or, if there are a mixture of AttB/AttP and AttL/AttR sites on the input documents, a BP reaction on all documents with AttB/AttP sites, followed by an LR reaction on the results of the BP reaction and any input documents with AttL/AttR sites. For example, to insert a PCR product with attB sites directly into a destination vector, select the PCR product, a donor vector, and a destination vector. Geneious will first produce an entry clone from the PCR product and donor vector, then react this entry clone with the destination vector to produce an expression clone.

10.5 Gibson Assembly

The operation will generate sequences with compatible overlaps that can ligate to each other after a partial chew-back with a T5 exonuclease. The overlaps are created by extension overlap PCR, the corresponding primers therefore will automatically be generated and displayed in a report document and as annotations on the resulting sequences. To enable Gibson Assembly you have to select two or more linear sequence documents or grouped documents. All necessary options are easily accessible from the interface (Figure 10.4).

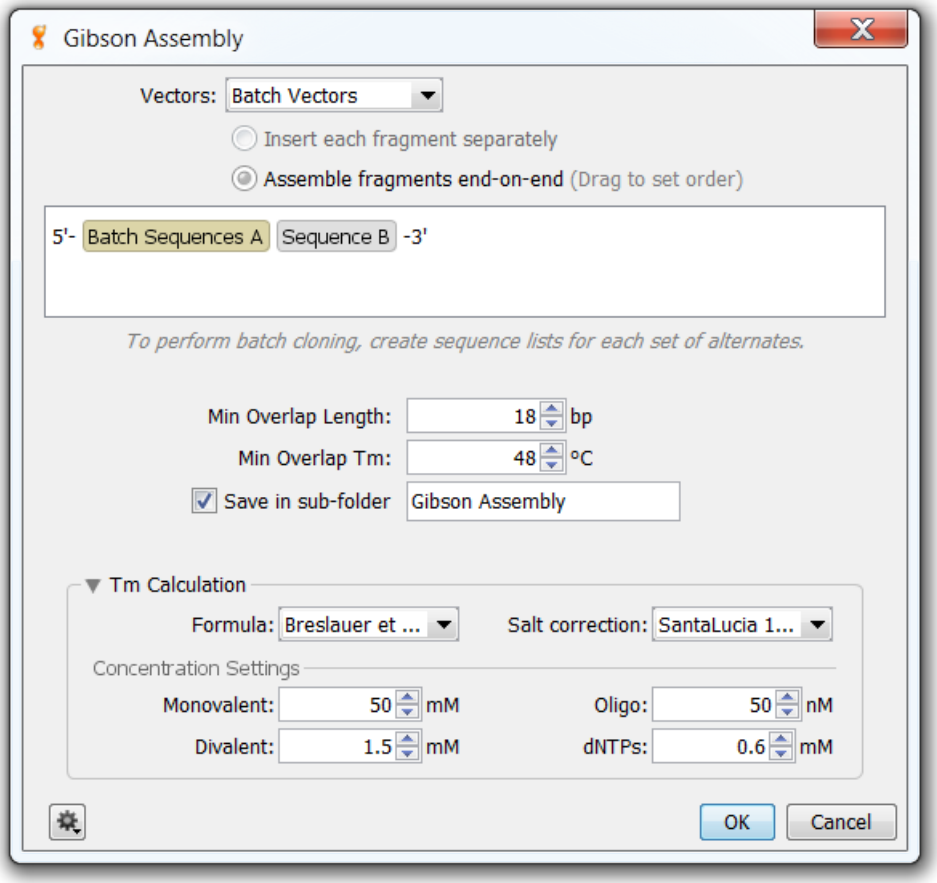

Gibson Assembly
Vectors: Batch Vectors
Insert each fragment separately
Assemble fragments end-on-end (Drag to set order)
5'- Batch Sequences A Sequence B -3'
To perform batch cloning, create sequence lists for each set of alternates.
Min Overlap Length: 18 bp
Min Overlap Tm: 48 °C
Save in sub-folder Gibson Assembly
Tm Calculation
Formula: Breslauer et ...
Salt correction: SantaLucia 1...
Concentration Settings
Monovalent: 50 mM
Oligo: 50 nM
Divalent: 1.5 mM
dNTPs: 0.6 mM
OK
Cancel

Figure 10.4: *Gibson Assembly* options dialog, with extended options showing.

- A dropdown menu provides easy access to chose a vector. If 'none' is selected the product(s) will be linear, otherwise circular. The longest sequence will automatically be preselected as vector.
- Insert sequences can be ordered via drag-n-drop. Sequences that have been previously

grouped into lists (Batch Sequences A, shown in brown-ish) will all be inserted at the specified position, generating one product per sequence at that position.

- If no grouped documents are provided in the drag-n-drop field the user can chose to insert the sequences either all at once into the vector as a sequential assembly (Assemble fragments end-on-end), or alternatively as alternating inserts with one insert per generated product (Insert each fragment separately).
- The minimum overlap (length of the complementary sequence) can be specified as well as the minimum melting temperature of the complementary sequence (Min Overlap Tm).
- To calculate the Tm a collapsible field is available showing the options provided and required by `primer3`.

The operation will remove 5 ′ overhangs and fill up 3 ′ overhangs that are eventually present on the sequences, eg. when they derived from a restriction digestion. The possible different sequence combinations are created and complementary extremities that might be already present will be considered when primers get designed.

For sequences without complementary overlaps a pair of primers will be generated. If both or only one of the ends are complementary, primers for both ends will be created, since the sequence will still have to be modified by a Primer Extension PCR to make it compatible at the opposing end. If both are complementary no primers will be generated.

extensions will be added to the primer corresponding to the neighboring sequence. Thereby modifications that have been manually introduced at the extremities (annotations with the tag 'editing history') will be automatically added as part of the extension, so that they get introduced to the sequence during the PCR.

The melting temperature is calculated for the primer binding sequence and the extension part including the modified bases. For both, primer and extension generation, the user specified Tm formula is used. In many cases the Phusion DNA polymerase is used for which it is recommended to use the Tm formula of Breslauer et al. 1986 (`http://www.pnas.org/content/83/11/3746`).

Primers are generated only for insert sequences, supposing that the vector should stay unmodified. For this reason the extension length of the primer extending to the vector will be twice as long (the full specified minimal overlap length) compared to extensions on primers between two inserts who share half of the specified overlap length each.

For very short or long extensions `primer3` might fail to calculate a Tm. If the sequence is too short Formula 10.1 is used, if it has a length greater than 36bp Geneious uses the Formula 10.2.

$$Tm = 2[AT] + 4[GC] \tag{10.1}$$

$$Tm = 64.9 + 41\frac{[GC] - 16.4}{[ATGC]} \tag{10.2}$$

A Report Document will be generated listing the generated products and primers in a tabular view. Errors that occurred during the primer generation process will be reflected in that report document. Furthermore any modifications (recession or maintaining overhangs, adding modifications to primers) are shown at the beginning of the document.

Geneious has a built-in parent-descendant tracking system. Whenever a change is made to a parent sequence it will ask to propagate this change to its offsprings. However, in the case that the user introduced some changes in the primer binding region or the extension region of the original sequences, these changes won't be reflected in the report document.

10.6 TOPO® Cloning

TOPO Cloning lets you ligate a single fragment into a Vector within only 5 minutes using the natural activity of Topoisomerase I which recognizes a specific motif **5′ - (C/T)CCTT - 3′** on the DNA. TOPO is a registered trademark of Invitrogen Corporation.

With the option *TOPO Cloning* you can insert linear fragments into either linear TOPO vectors (when a TOPO-site is present at the extremities) or into circular TOPO vectors. You can select as many sequences at once as you like, they will be ligate into each other in a batch operation.

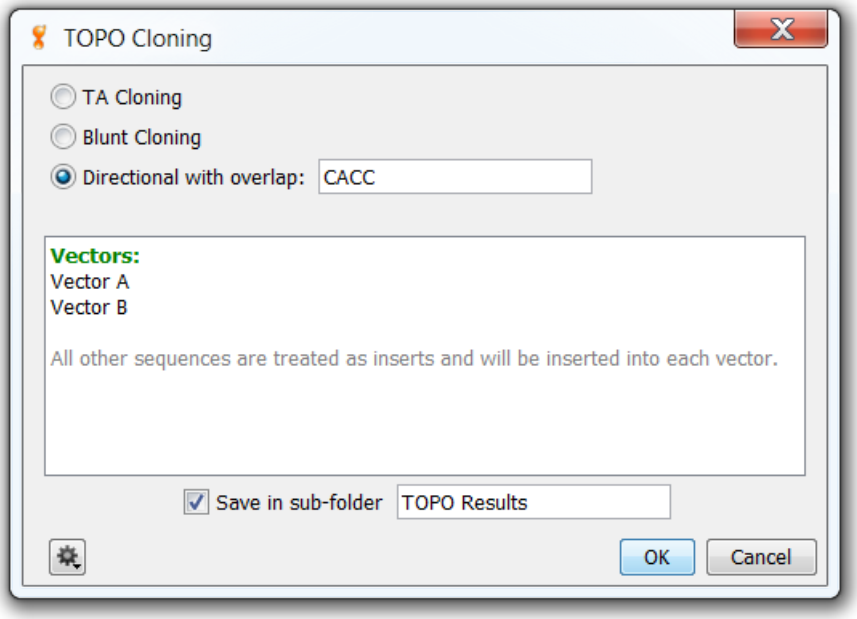


Figure 10.5: *TOPO Cloning* options dialog.

- Three different options (TA- Blunt- or Directional cloning) are shown on the top. If Direc-

tional is selected the user can define an overlap sequence. If this field is blank it has the same effect as Blunt cloning.

- The field below shows which of the selected sequences have been detected as vectors, all other sequences are inserts.
- If any complications occur, eg. when more than one TOPO site is detected or when a linear sequence with TOPO site is selected it will print a message in this box, also showing how the corresponding sequence is processed if the user clicks OK.
- The resulting sequences will be optionally saved in a sub-folder.

Chapter 11

Shared Databases

By using shared Databases Geneious can store your documents in your favorite relational (SQL) database rather than on the file system. This means that multiple users can concurrently use the same synchronized storage location without any problems.

A shared Databases can be used for everything a local database is used for. This includes collaboration. Take note that unread status, agents and shared folders belong to individual users rather than the database. For example Bob may see a document as unread, but Joe will see that same document as read if he has read it.

11.1 Supported Database Systems

To use a database as a shared Database Geneious requires that it support transactions with an isloation level set to SERIALIZABLE. Supported databases systems include Microsoft SQL Server, PostgreSQL, Oracle and MySQL. It is possible to use other database systems if you provide the database driver, see section 11.2.1

Shared Databases have been tested using:

- Microsoft SQL Server 2005 Express
- PostgreSQL 7.4
- Oracle 10g Express Edition
- MySQL 5

11.2 Setting up

After a database is set up correctly, multiple users can connect to it and use it as their storage location just as if they were using their own local database.

Follow these steps to set up your database for use with Geneious.

- Install a supported database management system if you do not already have one.
- Create a new database with your desired name. Make sure that you have a user that has rights to create tables.
- Use the "Connect to a database button" to connect to your database. If the database has not been set up (usually the case if you are following these instructions) Geneious will detect this and set up the database. This will only succeed if you have permission to create tables on the database.
- Make sure any other users of the database have SELECT, INSERT, UPDATE and DELETE rights, otherwise they will not be able to use the Shared Database as intended.

There are two ways you can use your database with multiple users. The simple way is just to use the Shared Database as a shared local database. If this is all you want then you are now done with setup.

Alternatively you may want to restrict access to particular folders with groups and roles. To do this please refer to section 11.4.1.

Your database should now be ready to use with Geneious. Now all users can connect to the database by clicking on Shared Databases in the service tree and then clicking "Connect to a setup database". This will bring up a dialog for the user to enter in the database details.

11.2.1 Supplying your own Database Driver

Shared Databases were designed with the supported databases in mind and packaged with database drivers for them. However Geneious allows you to supply your own jdbc database driver if you want to.

You may want to do this because you have an updated driver or because you have a driver for an unsupported database. It is not guarnteed that Shared Databases will work with another database system if you provide its driver, but it is likely that it will.

To supply your own driver open up the dialog you would normally use to connect to a database. Then click the 'More Options' button.

11.3 Removing a Shared Database

To remove a Shared Database, simply right click on its top folder and choose "Remove database".

11.4 Administration

The typical user will not have to do any administration, this section is for those in charge of the database.

11.4.1 Groups and Roles

Shared Databases support user groups and roles for managing access to documents. This means that you can restrict access of folders to privileged people. How it works is that each folder in Geneious belongs to a group. Users can belong to any number of groups and have a specified role within that group. The three roles are:

- "View" allows the user to view the contents of folders.
- "Edit" allows the user to view and edit the contents of folders.
- "Admin" allows the user special administrative functions on folders.

As of this time Geneious only uses the "Admin" role for the "Everybody" group.

By default there is only one group, the "Everybody" group. When a user logs in for the first time Geneious will put them into the "Everybody" group with a role of "Edit". So this means every user of the shared Database belongs to this group with a role of "Edit" unless you enter them into the "g_user" table beforehand. You will want to give yourself the role of "Admin" for the "Everybody" group if you want to perform administrative functions within Geneious.

Unfortunately at this time there is no interface for assigning groups and roles to users. So you will need some knowledge of SQL in order to take advantage of this feature. You can create groups by adding entries into the "g_group" table in the database. Assign users groups and roles in the table "g_user_group_role".

It is likely that if you are running in a multi user environment and taking advantage of groups and roles you will want to give only read-access of the table "g_user_group_role" to your users. This is so your users can not edit this table with SQL directly as you would do. You will also want to add all of your users into "g_user" manually so Geneious does not think that they are first time users and fail trying to insert them into the "Everybody" group due to read-only access.

Chapter 12

Licensing

The Help menu contains a number of features controlling the use of your Geneious license(s).

12.1 Activate License

This item lets you activate a license, or choose to connect to a license server. The options are as follows:

- *Use license key*. If you have purchased a personal license you can enter the details here to activate it. Make sure you enter the licensee name exactly as it appears in the email in which you received your activation ID/registration key. An internet connection is required to activate personal licenses.
- *Use license server*. If your organization has purchased a floating license administered through a FLEXnet license server, this is where you enter the details required to connect to the license. Ask your system administrator for the host name and port of the license server.
- *Use Sassafras KeyServer*. If your organization has purchased a floating license administered through Sassafras KeyServer, select this option. Your system administrator needs to configure KeyAccess to point to the KeyServer license server.

12.2 Install FLEXnet

This installs the FLEXnet license manager which is necessary for activating a personal license. When you try to activate your license Geneious will tell you if this is necessary. Only an administrator on your computer can do this but it only needs to be done once from one user account.

Once this has been done, any non-admin user can activate their license on the machine. The admin should not activate licenses for users as this will prevent the user from activating the license themselves.

12.3 Borrow Floating License

This item is only available to users for a floating license administered through a FLEXnet license server. Borrowing a license allows you to borrow one of the seats of a floating license so you can use it even when disconnected from the network. Since this decreases the number of seats available for other users, borrowing can only occur with the authorization of the system administrator. If your borrowing is approved, the system administrator will provide you with a ”borrow file” authorizing the borrow. To borrow a license, check ”Borrow” in the menu, and navigate to this file when prompted by Geneious. Borrowed licenses have an expiry date, when they will automatically be returned to the server, but if you are finished with the license before the expiry date, please uncheck ”Borrow” in the menu while connected to the network in which the license server resides, so that the license is returned to the server and is available to other users again.

12.4 Release License

Personal licenses can only be activated on a maximum of three computers simultaneously. If you no longer need to have Geneious available on a computer where you have activated it, you can release the license so it is available for use on another computer. Licenses cannot be released too often so do not do it unnecessarily.

If you’re using a floating license, you can release it allowing another user to access it without you having to shut Geneious down. Once you’ve released the license, Geneious will enter restricted use mode.

12.5 Buy Online

This item will open the Geneious store in your browser.

Chapter 13

Geneious Server

13.1 Introduction to Geneious Server

If your site has a Geneious Server installed you can use it to offload many of the tasks that Geneious would normally run locally on to the server, taking the processing load off your own computer. Once a job is sent to Geneious Server, it will either be processed on the server itself (a so-called standalone installation) or be handed off to a cluster running Oracle Grid Engine, LSF or PBS schedulers.

To use Geneious Server, a server-side user account is required. The server-side user account will have a server access license associated with it. Another possible configuration is that your server may have a queue licencing system, which allows a certain number of users to run jobs on Geneious Server simultaneously.

If your user account has its own access license (GSAL) then you can connect to the server and execute jobs immediately without having to wait for a queue license to become available. If your account doesn't have an access license then you can log in and submit the job to the server where it will join the queue and execute when a queue license becomes available.

13.2 Accessing Geneious Server

Assuming you have your account configured on the server, you'll need to install the necessary Geneious Server plugins. Many of your normal Geneious plugins are already server aware but there are other plugins which are different from the standard plugins, or are Geneious Server exclusive as they offer features unique to Geneious Server.

Your administrator can provide you with a download location for Geneious Server plugins. You can get them either from the Geneious Server itself or they may be hosted on a network

location with the `.gplugin` files. If you have the plugin files, just drag them all into Geneious. If you have to go to the web interface, get the URL from your administrator and you should see a page like figure 13.1.

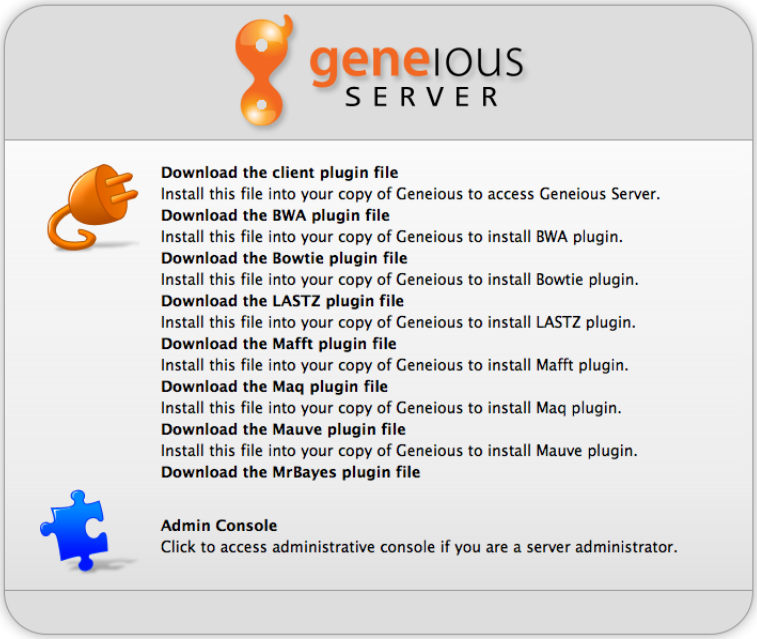

geneious
SERVER
Download the client plugin file
Install this file into your copy of Geneious to access Geneious Server.
Download the BWA plugin file
Install this file into your copy of Geneious to install BWA plugin.
Download the Bowtie plugin file
Install this file into your copy of Geneious to install Bowtie plugin.
Download the LASTZ plugin file
Install this file into your copy of Geneious to install LASTZ plugin.
Download the Mafft plugin file
Install this file into your copy of Geneious to install Mafft plugin.
Download the Maq plugin file
Install this file into your copy of Geneious to install Maq plugin.
Download the Mauve plugin file
Install this file into your copy of Geneious to install Mauve plugin.
Download the MrBayes plugin file
Admin Console
Click to access administrative console if you are a server administrator.

Figure 13.1: Download Geneious Server Plugins

Click on each plugin to download it and once you've downloaded all plugins, drag them from your downloads folder into Geneious. You'll probably have to restart Geneious after all plugins have been installed. Note that it may take some time for the plugins to install so give it some time. Once it is clear the plugins have all installed, restart and when Geneious comes back up you should now see the Geneious Server link in the Sources Panel. Click this and you'll see a button to log in. Use the log in button to display a dialogue requiring the hostname, username and password details which your administrator should have provided you with (Figure 13.2)

Once you've logged into the server, you will now have access to the shared database space which will appear under 'Shared Databases' in the sources panel. We recommend you create a folder for your own documents. The benefits of this folder is that the server can see anything in there without having to get it from your Geneious client. This means large documents such as NGS sequencing data can be placed in here and the server will be able to quickly access it. Also, if you log into the server from another machine, documents you put in the shared Database will be available unlike those of your local database. You can also see other users' data so this is a good way to share your documents. This is exactly like the normal shared Databases available with Geneious, but this database is preconfigured and available as soon as you log into the server. Don't try and access it any other way using the normal shared Database plugin.

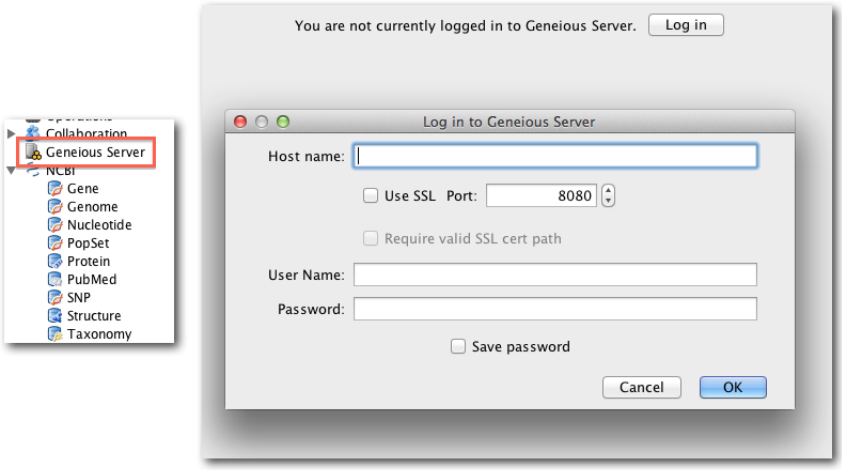


Figure 13.2: Log in to Geneious Server

13.3 Running jobs and retrieving results

Once you've logged into Geneious Server, many normal operations will now include an additional pair of buttons indicating whether the job should run on your computer or on Geneious Server (Figure 13.3). Whenever you see this choice you can choose to run the job on Geneious Server. If you're not logged in when you choose this, Geneious will prompt you to log in. The rest of the options are the same as for any local job, and the job will progress in the same way as if run locally, only using the remote resources provided by the server. If the job is likely to complete quickly, you should just run it locally but if it requires a lot of memory (more than your local machine has for instance) or if it will take a long time to process you should choose to run it on the server.

You can check the status of your job in the operations table in Geneious and you can also shut Geneious down once your job has been submitted to the server and if the job has completed when you log back in you'll be able to retrieve your results. If your jobs were running when you shut down, Geneious will request progress from the server when you restart and either show you your completed jobs, or show you the progress dialogue so you can see how far along the job has gone (Figure 13.4).

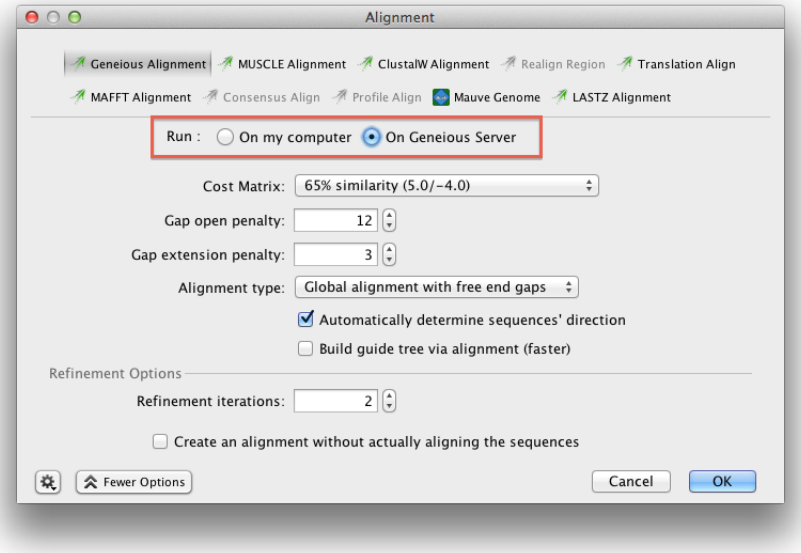


Figure 13.3: Log in to Geneious Server

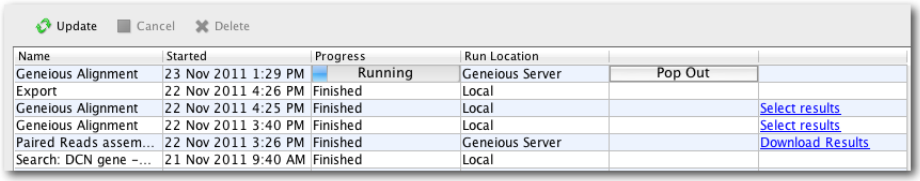


Figure 13.4: Operations table showing Geneious Server and local jobs

13.4 Geneious Server enabled plugins

This table details plugins which work with Geneious Server. Note that some of these plugins only run on Geneious Server so if you try and run them locally you will get a warning that this is the case.

Plugin	Local	Server
Geneious Alignment	Yes	Yes
MUSCLE Alignment	Yes	Yes
ClustalW Alignment	Yes	Yes
Realign Region	Yes	Yes
Translation Align	Yes	Yes
MAFFT Alignment	Yes	Yes
Consensus Align	Yes	Yes
Profile Align	Yes	Yes
Mauve Genome	Yes	Yes
LASTZ Alignment	No	Yes
Geneious Tree Builder	Yes	Yes
Consensus Tree Builder	Yes	Yes
MrBayes	Yes	Yes
PHYML	Yes	Yes
PAUP*	Yes	Yes
Geneious Assembler/Mapper	Yes	Yes
Bowtie short read mapper	No	Yes
BWA short read mapper	No	Yes
Maq short read mapper	No	Yes
SOAP2 short read mapper	No	Yes
Tophat RNAseq aligner	No	Yes
Velvet short read assembler	No	Yes
Find Variations/SNPs with SAMtools	No	Yes
CustomBLAST	Yes	Yes

Chapter 14

Administration

14.1 Default data location

By default, the data location will be in the user's home directory. You can change this by setting an environment variable which will be used by the Geneious launcher such as setting a `$HOME$` variable to be where you want a user to store their data.

On Windows and Linux, edit the `Geneious.in.use.vmoptions` file in the installation directory, and add `-DdataDirectoryRoot=$HOME$/Geneious` on a new line after the other settings.

On Mac OS X, edit the `/Applications/Geneious.app/Contents/Info.plist` and find the `<key>Arguments</key>` section to match the following:

```
<key>Arguments</key>
<string>-distributionVersion
-DdataDirectoryRoot=$HOME$/Geneious</string>
```

A special `$JAVA_USER_HOME$` variable is normally used which resolves to `user.home` and is what Geneious uses by default. The program will create a `Geneious 7.0 Data` folder inside the directory you specify.

14.2 Change default preferences

14.2.1 Change preferences within Geneious

Start a fresh copy of Geneious, set it up the way you want. Shut down and then copy `Geneious`

`7.0 Data/user_preferences.xml` to the Geneious install directory (e.g. `C:\Program Files\Geneious` on Windows XP) and rename it to `default_user_preferences.xml`

Now, when users start Geneious for the first time, they will get the configuration you set rather than the normal default.

Examples of features you can change:

- Turn off automatic updates
- Set default custom BLAST location
- Set up a shared Database
- Set up a proxy server default
- Turn off particular plugins

Any users who have already run Geneious should click the "Reset All Preferences" button in the Geneious Preferences to load these defaults.

14.2.2 geneious.properties file

Any preferences which can be set within Geneious can also be set from the geneious.properties file which can be found in the Geneious installation directory. Some examples are present in the file already- remove the hashes from the start of the lines and modify the values to use them. If you need to find out how to set other preferences using this file, please use the Support button in the Geneious toolbar to request help.

14.3 Specify license server location

Create a plain text file in the Geneious installation directory called `server.txt` that has the hostname on the first line and the port on the second line.

14.4 Deleting plugins

Features of Geneious can be turned off in preferences so the section on changing default preferences would be the simplest solution. However, if you really want to delete a feature completely so your users can't reinstate it you should shut down Geneious, go to the installation directory, into the `bundledPlugins` directory and delete the desired plugin jar files/folders.

14.5 Max memory

On Windows and Linux, edit the `vmoptions.current.defaults` file in the installation directory and change the `-Xmx` value to your preferred setting.

On Mac OS X, edit the `/Applications/Geneious.app/Contents/Info.plist` file and find the `VMOptions` section and modify the `-Xmx` setting.

It is important on Mac OS X to ensure that this value is set appropriately after an upgrade because users can often find that they have many large files in their local database preventing Geneious from starting if this value is reset to the normal default (700M on 32 bit, 1000M on 64 bit). This is an issue because the `Info.plist` file is stored in the Geneious app bundle so it gets replaced when upgrading.

Chapter 15

Troubleshooting

15.1 Local database issues

This section will help you deal with typical issues with the local database.

15.1.1 The local database

Geneious stores the user's data in a folder called `Geneious 7.0 Data` which will be located in the user's home directory by default. When you upgrade, Geneious offers to create a copy of this folder (with the upgrade's version number in the name) and update the format.

Geneious databases are not backwards compatible so if you upgraded and haven't accepted the offer to keep a backup you will not be able to downgrade. If you downgrade to an earlier version, you won't be able to see documents you created in the newer version.

15.1.2 Storing the database in a non-standard location

If you want to access your data from multiple computers, these are **not** the way to do it:

- Don't store local database on a network drive
- Don't use a tool like DropBox to sync the database

Storing data on a network drive can lead to very poor performance because Geneious accesses the database frequently so we do not recommend this. A typical problem would be documents that don't show up in the document table immediately or changes to documents don't persist. Windows Vista and 7 have also had issues where they change ownership of documents when

accessed from other machines and this prevents the user from changing them from a different login.

Storing data on a syncronising service is not recommended because the changes to the Geneious database need to be completely copied to the remote service for it to remain intact. Since outgoing connections can be quite slow it is too easy for the sync to be cut short and then when the other computer tries to sync with the remote service the local database is corrupted.

Users who must access data from multiple places should use:

- A USB drive that they can put documents on in `.geneious` format which can then be dragged into another local database on another machine. In theory you could put your entire local database on the drive but this could result in permissions issues mentioned earlier so isn't recommended
- Put the `.geneious` files on DropBox or similar, but definitely not the entire local database.
- Access a Shared Database which will handle the transactions correctly and is the best solution all around to accessing data from multiple sources

15.1.3 Sharing files or the local database

It isn't unusual to want to share files with other users. Geneious has a simple Jabber client which can do this but users all need to be running Geneious at the same time for the files to be accessed. To get around this we have seen examples where users have shared a single local database. This is a very bad idea as there is no file locking and users can harm each other's data. Permissions on Windows Vista and 7 can also cause unpredictable behaviour such as inability to modify files.

The solution is for users to have their own local database and to access shared content via a shared Database, or to export documents in `.geneious` format to a shared drive for others to access.

15.1.4 Lost data

This can happen when you have upgraded multiple times since you may have had issues finding your data so could have ended up loading older databases. In these cases, data for Geneious 7.0 may actually have been stored in the `Geneious 4.8 Data` folder for example. The trick is to identify which of potentially multiple local database folders your most recent data was in. Date stamps on the folder should help in this respect (Figure 15.1).

In **Preferences** → **General** tab (Figure 15.2) you can browse to the location of the last local database you accessed and Geneious will switch and import the data that is there. If you have

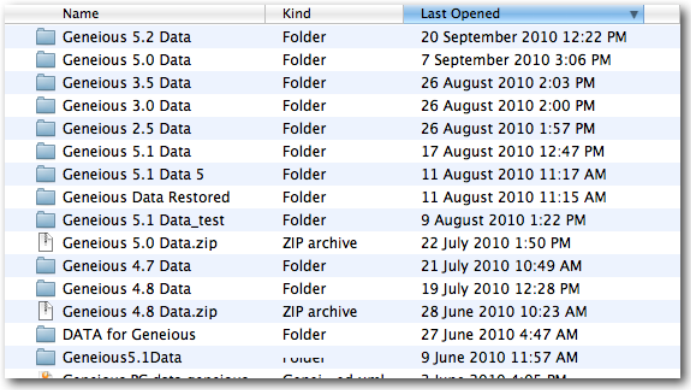


Figure 15.1: Sorting data folders by date

found your data it is a good idea to use **File → Back Up Data...** to save the documents in a format that can then be loaded into Geneious again. You may even want to tidy up a bit and delete old data folders if there are lots of them.

It would be better if you make regular backups so we encourage you to do so.

15.1.5 Backing up the local database

You should be aware that you need backups. Due to the way the local database works, it is important that Geneious is not accessing the database when a backup is taken. For example, Mac users with Time Machine will have backups taken during the day but if Geneious is running when those backups are taken, they will not be suitable for restoring from and Geneious likely wouldn't start if you did. In that case, backups taken overnight when Geneious isn't running would be fine though.

There is a backup button (Figure 15.3) which will cause Geneious to cease working on the local database and make a zip archive. You should use this regularly and the backups should be stored on another drive, or can be left to general system backups safely since these are made when Geneious is in a non-running state. These backups can also be safely moved around including to other machines.

15.1.6 Moving to another computer

It is normal for IT people to move users from one computer to another while having little knowledge of the applications and data that they're moving. Before you hand over your machine, you should make a backup of your data. IT may just use Explorer on Windows to move

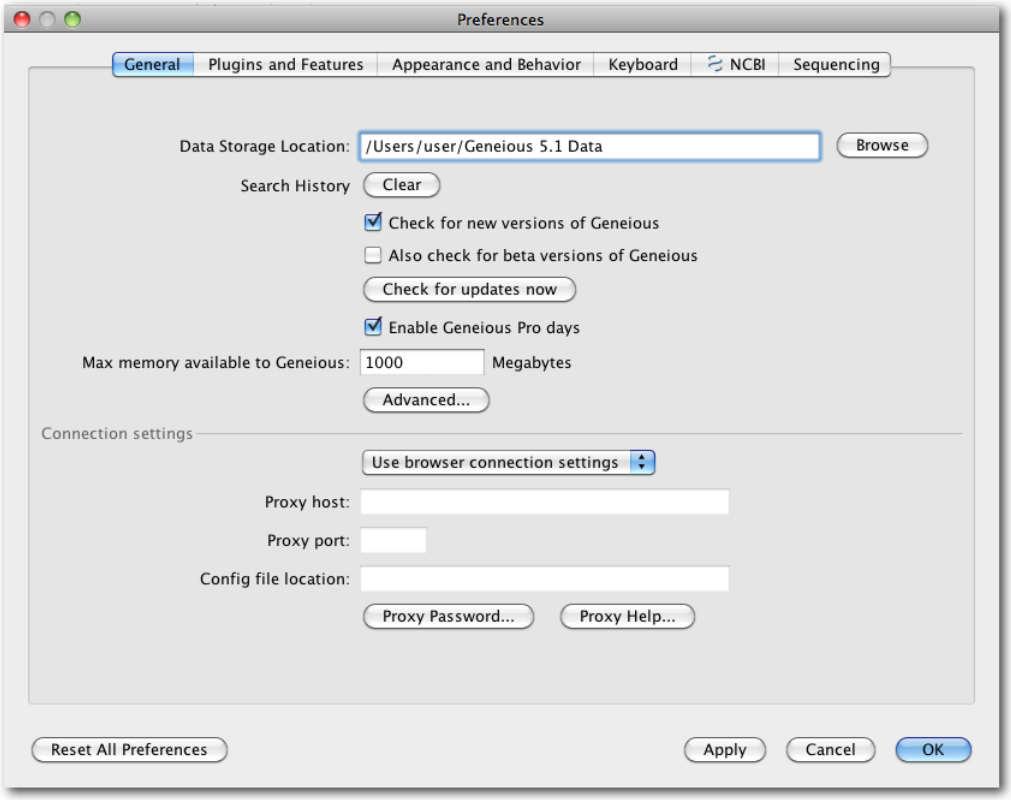


Figure 15.2: Setting the local database location

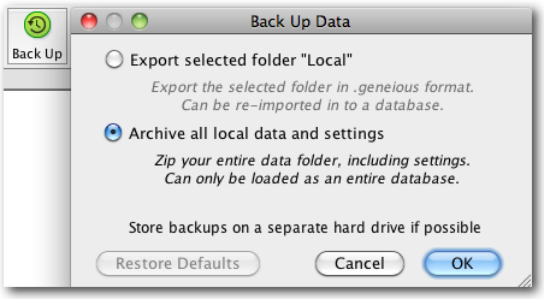

Back Up
Back Up Data
Export selected folder "Local"
Export the selected folder in .geneious format.
Can be re-imported in to a database.
Archive all local data and settings
Zip your entire data folder, including settings.
Can only be loaded as an entire database.
Store backups on a separate hard drive if possible
Restore Defaults
Cancel
OK

Figure 15.3: Using the backup tool

your files from the old machine to the new one but this will break the Geneious local database because files and paths are longer than the maximum 256 bytes that Explorer handles so files will get lost.

The backups that Geneious produces can be safely moved so even if IT does this, the data can be restored from the backup. It is also necessary to release a license before moving machines. This can be done from the 'Help' menu. Note that there are a limited number of releases available within a given period of time and trying to release too often may be misconstrued as a user trying to share a personal license with others. Only release a license when absolutely necessary.

15.1.7 Reinstalling Geneious won't erase user's data

Because the Geneious installation isn't in the same place as the user's data, you can safely uninstall Geneious and your data will be untouched. When upgrading, it is cleaner to uninstall the previous version before installing the new version. While upgrading over the top usually works, there have been issues due to permissions that have prevented it so uninstalling is needed to work around these.

15.2 Network issues

15.2.1 Connection error when trying to search using NCBI or EMBL

If the message reads, "Check your connection settings", there is a problem with your Internet connection. Make sure you are still connected to the Internet. Both Dial-up and Broadband can disconnect. If you are connected, then the error message indicates you are behind a proxy server and Geneious has been unable to detect you proxy settings automatically. You can fix this problem:

1. Check the browser you are using. These instructions are for Explorer, Safari, and Firefox.
2. Open your default browser.
3. Use the steps in Figure 15.4 for each browser to find the connection settings.
4. Now go into Geneious and select "Preferences". There are two ways to do this.
   - *Shortcut keys*. Ctrl+Shift+P (Windows/Linux), ⌘+Shift+P (Mac OS X).
   - *Tools Menu → Preferences*.
5. This opens the Preferences. Click on the 'General' tab. There are five options in the drop-down options under "Connection settings" (Figure 15.5):
   - *Use direct connection*. Use this setting when no proxy settings are required.

- *Use browser connection settings*. This allows Geneious to automatically import the proxy settings. This may not work with all web browsers.
- *Use HTTP proxy server*. This enables two text fields : Proxy host and Proxy port. This information is in your browser's connection settings. Use this if your proxy server is an HTTP proxy server. Please see step 3.
- *Use SOCKS proxy server - Autodetect Type*. This enables two text fields : Proxy host and Proxy port. This information is in your browser's connection settings. Use this if your proxy server is a SOCKS proxy server. Please see step 3.
- *Use auto config file*. This enables one text field called "Config file location". These details can also be found in your browser's settings.

6. Set the proxy host and port settings under the General tab to match those in your browser.
7. If your proxy server requires a username and password you can specify these by clicking the 'Proxy Password...' button directly below.

Note. If you are using any other browser, and cannot find the proxy settings, please use the Support Button in the Geneious toolbar to contact Geneious support.

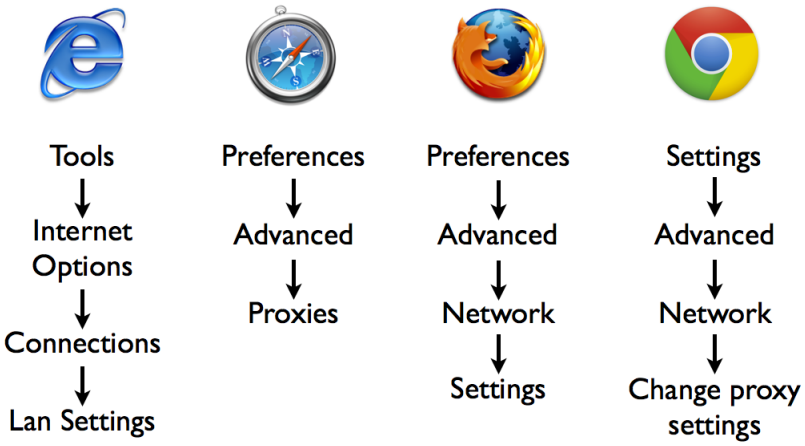


Figure 15.4: Checking browser settings

15.2.2 Web links inside Geneious don't work under Linux

Set your `BROWSER` environment variable to the name of your browser. The details depend on your browser and type of shell.

For example, if you are using Mozilla and bash, then put `export BROWSER=mozilla` in your `~/.bashrc` file. When using a csh shell variant, put `setenv BROWSER mozilla` in your `~/.cshrc` file.

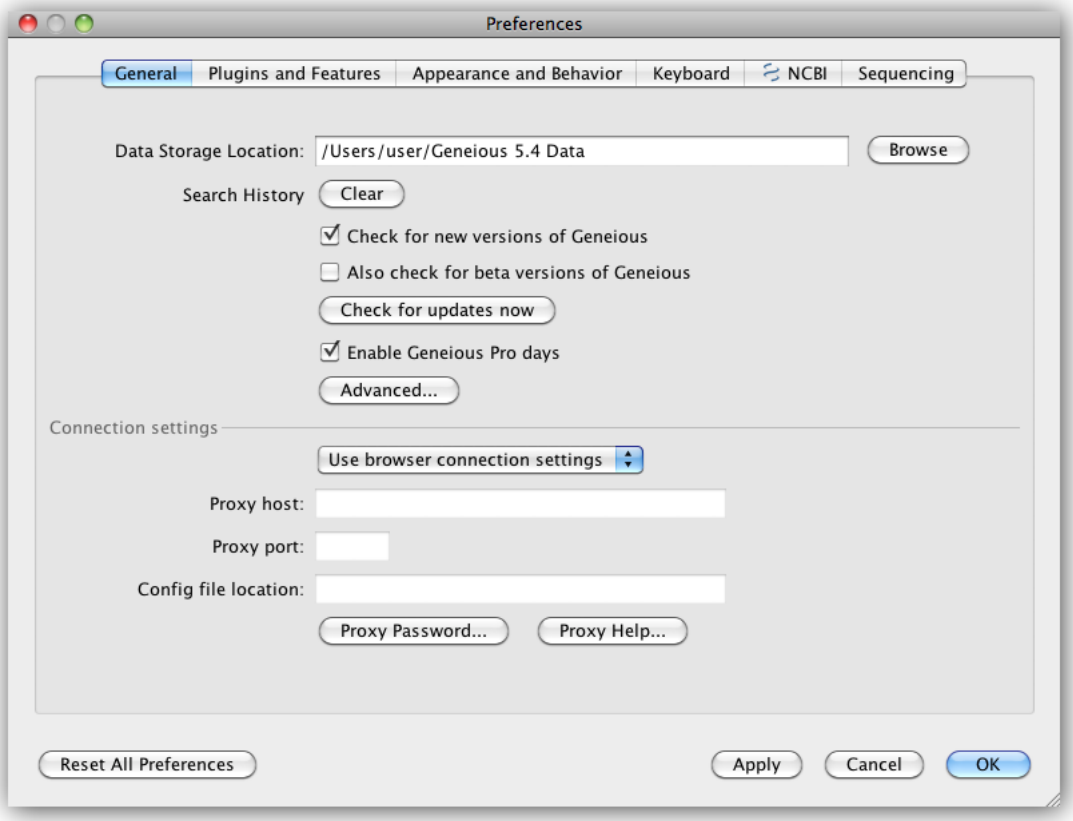


Figure 15.5: General Preferences

15.3 Geneious is slow

Geneious has pretty high memory and CPU requirements. It is becoming increasingly impractical to run it on 32 bit hardware since the realistic upper limit for memory that can be dedicated to Geneious is 1GB on those machines. With that said, there are things that can be done to improve the performance of Geneious even on limited machines.

15.3.1 Memory

Geneious runs in a Java Virtual Machine. When this JVM starts, it will be allocated a certain amount of RAM and the program can use less than that but never more.

In the **Preferences** → **Appearance and Behavior** tab, there is a button to turn on the memory usage bar (Figure 15.6). This is well worth doing as it will show how much RAM Geneious has available and how much it is using. Also, clicking this bar (which will appear under the 'Sources' panel) will force a garbage collection freeing up memory within the JVM.

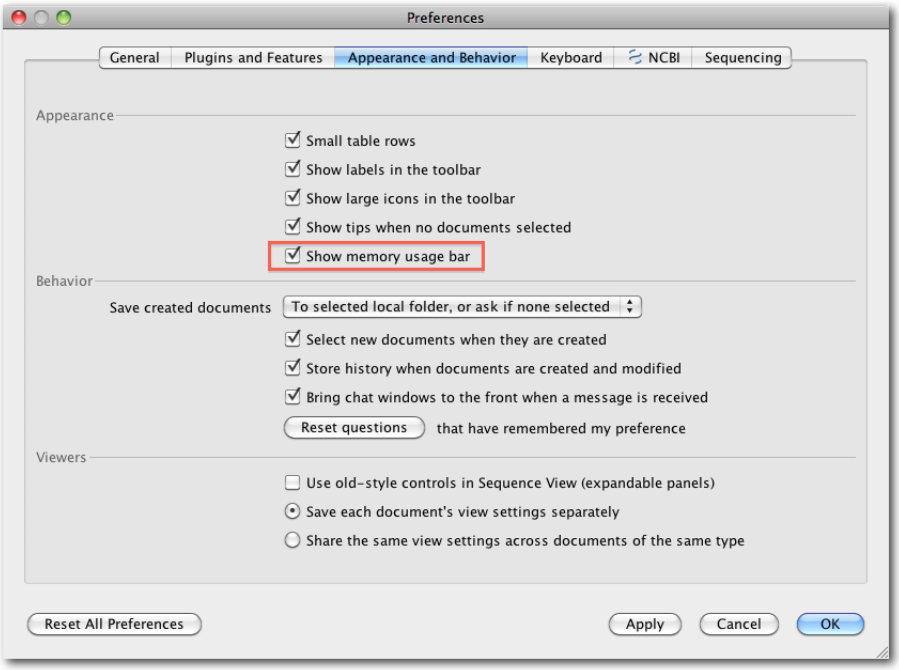


Figure 15.6: Turn on memory usage bar

While it may be tempting to allocate more memory to Geneious, bear in mind that the operating system and other programs cannot use this RAM once the JVM is so if you allocate too much then everything will go much slower.

To be able to reliably allocate more than 1GB of RAM you need a 64 bit machine. If you have a 64 bit machine with a 64 bit OS installed and at least 4GB of RAM, you can safely allocate 2GB. If you have more RAM, then you can allocate more to Geneious. It isn't advisable to allocate much more than half the available memory because again you'll starve the operating system of resources.

Users will often complain that Geneious is using an huge amount of memory because they've looked at Task Manager on Windows, or Activity Monitor on a Mac (Linux users may well be more savvy in this respect) but the best way to see how much memory Geneious is really using is to use the memory usage bar. The JVM itself will use memory and it is the total RAM allocated to the JVM that users will see from the various monitors.

15.3.2 Indexer issues

Geneious uses the Lucene indexer as the basis of the searching function. The indexer has the ability to be paused so if you see the indexer running like mad, click the indexing indicator under the 'Sources' panel which shows it is indexing and it will pause (Figure 15.7). This may take pressure off the hard drive which can badly affect performance because if you have multiple applications that are thrashing the drive then everything suffers. Pausing the indexer can help get those other tasks finished and once they're done, the indexer can be restarted. If you don't restart the indexer, features such as enzyme lists, or test with saved primers may not function correctly or at all.

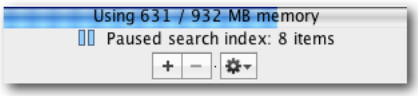


Figure 15.7: Pausing the indexer

It is possible for certain really large documents to cause the indexer to crash so if you hover your mouse over the indexing indicator, it will identify the document that caused the problem in a tool tip. Delete that document (export it to a safe place if you want to keep it) and then restart Geneious and the indexer should finish and go quiet.

15.3.3 Alignments take a long time

Although this is an operation, it can be seen as a 'Geneious is slow' issue because users often choose the wrong alignment tool and complain about performance. The standard Geneious aligner is based on dynamic programming and will be slow when presented with long sequences or large sets of sequences. In the case of large multiple alignments, you should look at

MUSCLE or MAFFT rather than the standard Geneious aligner. These are much faster and still quite accurate in most cases.

Some users have also tried to align genomes but this is bad because it will be horrendously slow, use an huge amount of memory (and usually crash as a result) and the end result is likely to be very poor simply because genomes tend to have inverted and duplicated regions which a traditional pairwise aligner won't cope well with. The Mauve Genome Alignment plugin exists for this purpose (Figure 15.8).

Figure 15.8: Alignment of genomes with Mauve

Another operation users try to do which can be very slow is to try and align many primers against a set of sequences. The right tool is 'Test with Saved Primers' but this can also be really slow if they have high levels of degeneracy and lots of sequences. The section on primers will offer potential solutions.

15.4 Importing and exporting data

Getting data into Geneious from other programs, and out for publication or use with other programs is generally easy but there are a few frequent issues.

15.4.1 FASTA file format

FASTA is simple and ubiquitous. It is also confusing to users and misused. The structure of a FASTA file is like this:

```
>Name Description
ATGTCGATGCAT
```

Users often mistake the description for the name, or wonder why their name is truncated when they imported it into Geneious when they have used spaces within what they consider the name. The name must not have spaces and if it does, they should be replaced with something like an underscore (_) to keep the name as a single item. The underscores can always be removed using 'Batch Rename' once the files have been imported.

15.4.2 Batch rename

Often when data has been imported into Geneious, the naming isn't what you expected. You should try the **Edit** → **Batch Rename...** tool which can replace any field with combinations of other fields, new text, and can also perform regular expressions to achieve very complex renaming operations.

15.4.3 Protein or Nucleotide

Most often, this happens with FASTA format since it doesn't declare what the data type is. When using drag and drop, Geneious tries to figure out what type of sequence it is looking at and use the correct import. To be certain that you've imported your data as the correct type though, use the **File** → **Import** → **From File...** and choose the format and type from the list. This will avoid embarrassing issues in the case of ambiguous data.

15.4.4 Word documents

Sequence data should never be stored in a word processing document. Word processors will do very odd things to file formats so if users want to use a document format to edit the data they should use a very simple text editor that can save text in UTF-8 (UTF-16 won't work so check the encoding your text file is saved in). There are many good choices. Just not Word.

15.4.5 Exporting data

Any export will likely lose some information. For an annotated sequence then GenBank format does a decent job of preserving the information in a form that many other programs will handle. However, if you want to preserve the look of the document, then you have to export the data as a graphic using **File** → **Save As Image File...**. Probably the most compatible is JPG but this is an image made up of dots so it is important to know this won't scale well. The default resolution will also be quite low so you should probably increase the resolution to about 400% to make the image look good when printed. For scaleable graphics, PDF or EPS are good choices. SVG is also scaleable and has the ability to be edited in tools such as Adobe Illustrator (expensive) or Inkscape (free). Using SVG will allow users to tweak the graphic, and add annotations and still have the image scale nicely because it is a vector graphic.

15.5 BLAST issues

Geneious allows users to run sequence searches using the NCBI BLAST service, or to install a local copy of BLAST to use with their own databases (CustomBLAST). Here are some issues you'll likely run into.

15.5.1 Can't connect to BLAST service

This is likely a problem with the proxy configuration. Geneious sends BLAST jobs via a URL on port 80 but if there is a firewall preventing direct access, then it will have to go via a proxy. Find out what the machine address and port are plus any user name and password necessary, and put those into the network settings in **Preferences** → **General** tab (Figure 15.9).

Figure 15.9: Proxy settings in Preferences

The implementation of the "use browser settings" may not work depending on the platform. On Windows, if the proxy is set in Internet Explorer it should work. Also, if a PAC file is

specified, Geneious will just grab the host address and port settings it specifies and use them to fill in the fields automatically.

15.5.2 Setting up BLAST for multiple users

The correct solution is to set up a WWWBLAST NCBI mirror locally and mirror all the BLAST databases as well as add some of your own. This will replace access to the NCBI service itself though. This may be too much for some people so they consider using CustomBLAST to achieve something similar.

One approach is to provide users with a set of sequences in FASTA format that they can create a CustomBLAST database from and keep that up to date and have them replace their local copies. This has the advantage that it is essentially purely parallel so it will scale indefinitely but it has the disadvantage that you can't be sure they're all searching the same database.

Since the Custom BLAST service access a folder on the user's hard drive, it is possible to put this folder on a share and have each user point at it. Their CPU will do the work but that data will be centralised. It is possible that this could cause performance issues over the network though and you'll need to deal with ownership and ensure that your users don't try adding databases themselves. You don't need to format the databases yourself from within Geneious but can use `formatdb` as normal to create BLAST databases and put them into the data folder. Geneious users will then be able to see them. You could also consider doing this with symlinks for some databases and then the users can create their own CustomBLAST databases while benefitting from your shared ones.

Note that if the database is formatted manually using `formatdb`, there will be no annotations on the resulting alignments. If it is formatted from within Geneious, then an extra file is created with the annotations so Geneious can put them back onto the alignments after a search.

15.5.3 BLASTing short sequences

Users should be aware that there are issues with BLAST when searching for short sequences. It is not guaranteed that it will find all occurrences of a short sequence in a database so users should not be surprised. Statistically, even with the word size set to 7 (the minimum for DNA searches) BLAST will miss 40% of possible hits when dealing with sequences of 20bp. This is why Biomatters has not implemented Primer BLAST. Users may want to use BLAST to test if primers match against their sequences because 'Test with Saved Primers' requires 5' extensions to be annotated so the test will ignore them, but this is also a bad idea since any matches it does produce will be local alignments rather than full length matches potentially truncating both ends, not just where the 5' extension is. It is possible to repurpose the assembler to do this though so see the chapter on primers.

If the primer has a 5' extension this should be annotated onto the sequence correctly and then

Geneious will ignore that region when primer testing. If this isn't done, the primer will not match. This would explain why some users have insisted that BLAST is the right tool for this job.

15.6 Primers

Primer design in Geneious is based on `Primer3` but the tool has been used in creative ways to perform many operations that it wasn't really designed for.

15.6.1 Primer testing performance is slow

When there are a lot of primers, the testing process can take a long time, especially when degenerate primers are being used or if you're testing primers as pairs. Testing as pairs can be especially slow with a lot of primers because Geneious has to test every possible combination and this can turn a task that should take seconds into one that will take hours so turn off the option to test as pairs if you don't need it.

Other programs have used BLAST to align primers against target sequences but this doesn't work well because BLAST is a local alignment tool so only the matching part of a primer will align so identity levels will not indicate the level of identity for the whole primer against the target sequence. Also, for short sequences, BLAST is not reliable so it will not be able to report all possible hits.

Geneious has a capable short read assembler which can handle mismatches and aligns the whole short read against the reference so it is possible to use this by selecting the sequences you want to test the primers against as the reference sequences (to do multiple sequences they need to be combined into a sequence list) and then selecting the primer sequences as the reads then doing a medium setting assembly. This will map all the primers that can match onto the references while retaining the regions that don't match (Figure 15.10). Note that it will reverse complement reads that match the other strand so you need to reference the primer annotation to see the primer sequence (Figure 15.11). If you want to check that primers don't match in multiple locations, be sure to switch to 'Custom Sensitivity' and turn on the 'Map multiple best matches' option in the 'More Options' section.

This method can be used as a quick screen to identify primers that will match your sequences and then you can use the actual primer testing tool.

15.6.2 Cloning Primers

When designing cloning primers it is necessary for the primers to be exactly at the ends of the CDS. This is essential for when doing Gateway cloning for instance. To do this, select the CDS

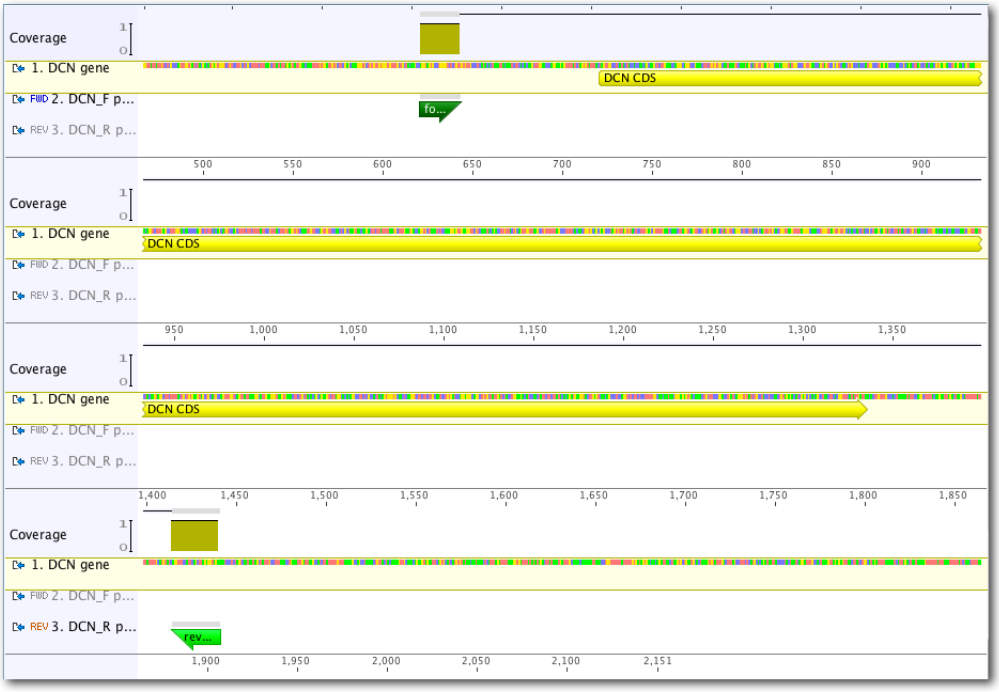


Figure 15.10: Using the assembler with primers

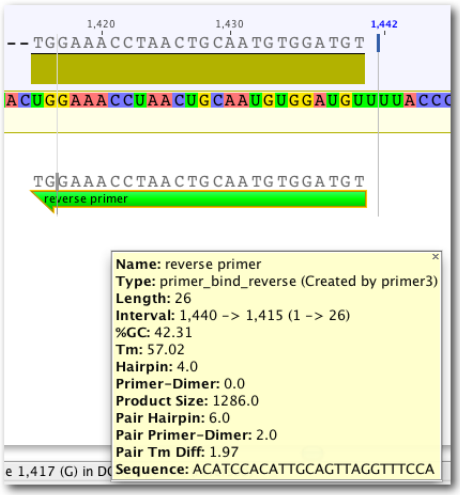


Figure 15.11: Reverse primer in assembly

you want to clone by clicking the annotation. Next, design new primers and turn off the target region and turn on the included region (which should be the CDS). Change the product size to be the length of the CDS (Geneious tells you this in the selected value shown at the bottom of the sequence viewer) in both boxes since it must be exactly the same length as the CDS. Only generate 1 pair. Since you're forcing the design to be in an area that may be less optimal you'll also likely have to drop the Tm minimum setting (Figure 15.12). When you hit OK the primers should be designed exactly at the ends of the CDS (Figure 15.13).

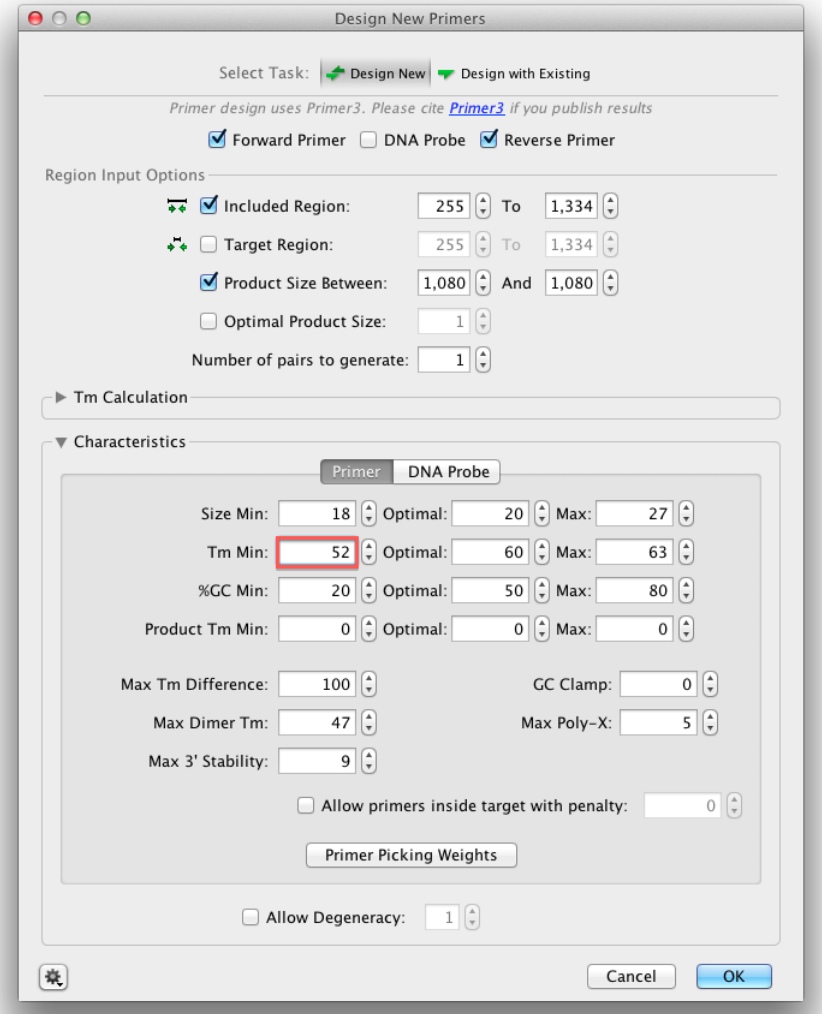


Figure 15.12: Design cloning primers

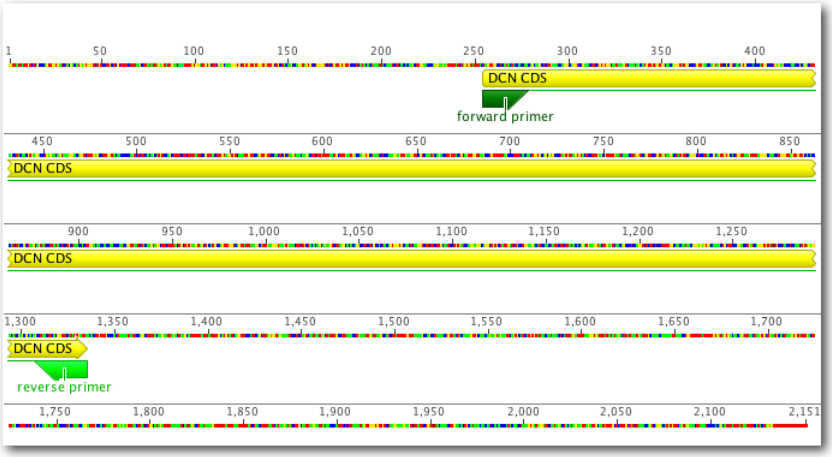


Figure 15.13: Finished cloning primers

15.6.3 Reverse complement primers

Primers are always 5′ to 3′ so in Geneious if you reverse complement a primer, the sequence viewer will show the other strand and the primer direction arrow will switch from left to right to right to left. In the text view you should see that the primer hasn't actually changed and is still the original sequence. If you really want to switch the primer to the other strand it needs to be run through 'Convert to Oligo' again since the annotated primer now doesn't correspond to the sequence. It is worth deleting the current primer annotations and then running the 'Convert to Oligo' tool which will create a new primer annotation running from left to right which does correspond to the sequence as it now exists.

This will create a sequence list which contains the primer sequences although they won't currently be oligos but you can then extract the sequences from the list and convert them to oligos using the **Primers** → **Convert to Oligo...** operation. They will now be available as part of the primer database.

15.7 Assembler

The assembler in Geneious has been written to be fast and memory efficient to allow it to handle next gen data. Here are some tips and tricks.

15.7.1 Trimming

Trimming in Geneious can be soft or hard. In the case of soft trims, the sequence will remain, but is ignored by many tools such as the assembler. This means soft trims can be adjusted as needed, or deleted completely. Soft trims can be confusing to users of other software because they can see the sequence in the assembly but the sequence isn't really contributing to the assembly and won't be part of the consensus sequence. Dragging the ends of the trim annotation will make the newly untrimmed sequence visible and part of the consensus (Figure 15.14).

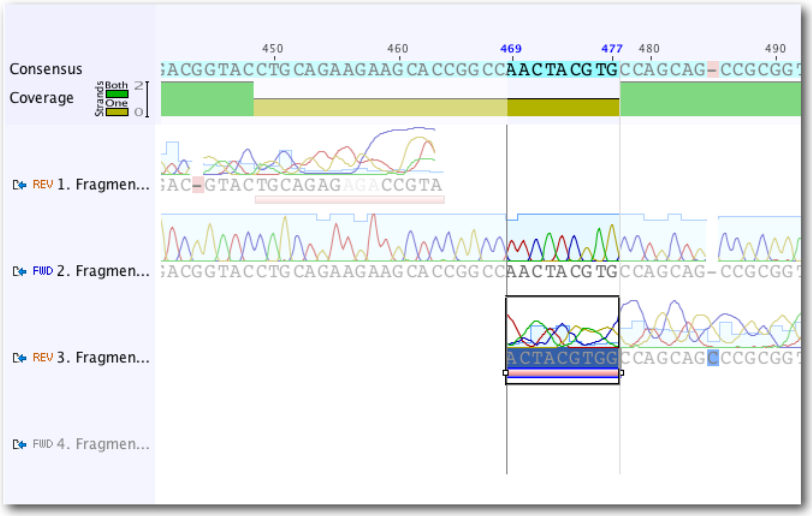


Figure 15.14: Click and drag the trims to adjust

15.7.2 Multiple reference sequences

The assembler can handle multiple reference sequences but they need to be combined into a sequence list document before assembly. Do this using **Sequence → Group Sequences into a List...** and then select this list as the reference sequence making sure Geneious will use all sequences. Geneious will then try all reads against all references in a single operation.

15.7.3 Paired reads

Paired read support is available but before it can be used, the read files need to be combined using the pairing operation. For example, if you have imported two FASTQ files, one with forward reads and one with reverse reads, you should select both and then use **Sequence → Set Paired Reads...** and choose the appropriate settings such as expected distance between

pairs. This will generate a new paired file which can be selected in the assembly operation and the extra information will be used to help the assembler resolve complex placement issues.

15.8 Installation and Licensing

15.8.1 Upgrading broke Geneious

If an upgrade has resulted in a broken install, uninstall Geneious and delete the Geneious installation folder (not the `Geneious 7.0 Data` folder though) and reinstall. This should fix the problem. There have also been some issues with the `user_preferences.xml` found in your `Geneious 7.0 Data` folder which can be solved by renaming it so Geneious creates a new one. If this wasn't the problem, you can rename it back without having lost all your preferences unnecessarily.

On a Mac, when you upgrade your memory gets reset to the default (this is due to how upgrades are handled on the Mac). Sometimes you have very large files in your local database which the default memory won't handle. To fix this, find the `Info.plist` file which is in the Geneious.app (right click to Show Package Contents and browse into the Contents and you'll find the file. Edit this and look for the `VMOptions` key. Edit the `-Xmx` value increasing the memory allocated to your previous value which worked and Geneious should now start.

15.8.2 Activation issues

Geneious has a FLEXnet based licensing system that requires on-line activation. The main issue with activation is if the program cannot access the licensing website. The address of this website is `http://licensing.biomatters.com` so if that site is blocked by a firewall then Geneious will be unable to register the license. Since this server is on port 80, it should be reachable but you may need to configure the proxy settings to enable access.

15.8.3 Admin license activation

Installing the license service requires administrator privileges. However, the admin should not activate the license because personal licenses are only available to one user on a machine and by doing so the actual non-admin user will not be able to use the license. If you must verify that the license works, make sure you release it using the Help menu item. Note there is a limitation on the number of times a license can be released to prevent license sharing.

FLEXnet licensing only needs to be installed once by the administrator after which, the user can upgrade Geneious as a non-admin.

15.8.4 Downgrading versions

When Geneious upgrades, it offers to create a new folder with the new version name and copy the data from the old data folder into this new one. This will mean you can downgrade if you prefer to use the earlier version, or if your license isn't able to run the latest version due to support expiry. Downgrading requires that the new version of Geneious is uninstalled first to avoid there being vestiges of the old copy in place. Once this is done, the old version can be reinstalled and Geneious will start up and see the old data folder but won't be able to access data created in the new version. If you have done work you need to get into the old version, you will need to export your data using an open format such as GenBank rather than just saving the `.geneious` format file prior to downgrading since Geneious files are not backwards compatible.

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